Metabolomics applied to biomarker discovery in liver related diseases

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Metabolomics applied to biomarker discovery in liver related diseases

by

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Abstract

The liver is the only human internal organ capable of regeneration. It is indispensable to most metabolic functions, such as bile production, metabolism of lipids, carbohydrates, proteins and amino acids, and breakdown of hormones. Since almost all other organs in the body rely on the liver for their smooth functioning, it is not surprising that the liver is closely related to a plethora of diseases. Liver failure, cholestasis and sepsis are common liver related disorders. A major drawback related to current biomarkers for liver disorders, such as aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and interleukins, is that they have limited diagnostic accuracy and specificity. Therefore, there is an urgent need to investigate metabolites that associate with different liver conditions, which can then potentially be utilised as biomarkers and therapeutic targets. The focus of my thesis was to apply metabolomics on human and mouse liver tissues to further understand its biochemistry under stress and facilitate the discovery of potential endogenous biomarkers under those conditions.

Chapter two involved an in-depth analysis of metabolic variations in two main types of liver donor tissues. Lipid fingerprints in 112 matched pre- and post-transplant liver biopsies from donation after circulatory death (DCD, n=36) and donation after brain death (DBD, n=76) were investigated using Reversed Phase-Mass Spectrometry (RP-MS). Two lysophosphatidylcholines, LysoPC (16:0) and LysoPC (18:0), showed higher levels in DCD, before transplantation ($q < 0.01$). These findings imply that the two LysoPCs play a role in perpetrating tissue damage induced by warm ischaemia. Interestingly, elevated amounts of these two lipids were also observed in recipients undergoing early allograft dysfunction (EAD) ($p < 0.05$).

After comparing DBD and DCD groups and identifying two potential lipid biomarkers of EAD, we decided to target lipids that are known to induce inflammation since ischaemia reperfusion injury (IRI) is associated with the release of pro-inflammatory mediators. In Chapter 3, a targeted analysis of five ceramides was carried out in 46 (10 DBD, 13 DCD) paired biopsies at both transplantation stages. It was found that C18, C20, C24 ceramides showed significant differences in DBD (p<0.05) pre- and post-transplantation, while C22 ceramide (p<0.05) showed more pronounced changes in the DCD group. A strong correlation between levels of C18 ceramide and bilirubin and creatinine, two common markers of liver dysfunction, suggested the involvement of C18 ceramide with IRI in DCD.

In Chapter 4, we aimed to extend the lipidomics results and investigated more polar molecules. Hydrophilic interaction chromatography-MS (HILIC-MS) analysis was performed on 35 DBD and 12 DCD paired liver samples (n=94). Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA) modelling suggested the involvement of five metabolites from the adenosine monophosphate (AMP) pathway. Elevated levels of AMP and adenine were indeed found in the DBD group, after univariate analysis (p<0.01). These changes were also seen in recipients experiencing immediate graft function (IGF) (p<0.05), suggesting that they may contribute to healthy allograft function and improve graft survival rate.

In chapter 5, an animal model of cholestasis was studied. Untargeted lipidomic analysis and semi-targeted bile acids analysis were applied to mouse livers (7 controls, 6 cholestatic) and plasma (8 controls, 10 cholestatic) from a cholestatic model. A panel of 5 lipids, including 2 phosphatidylcholines (PC (36:3), PC (34:2)) and 3 cholesterol

esters (CE (16:0), CE (16:1), CE (18:1)) showed significant differences between cholestatic and control mice (p<0.05). In addition, Receiver Operating Characteristic (ROC) curve revealed a high potential of these five lipids in discriminating cholestatic mice from controls, with area under the curve (AUC) of 100%, when compared to plasma enzymes (91.2%) and bile acids (77.5%).

Lastly, my focus moved to sepsis in order to understand inflammation in the liver and if this can affect the brain via the liver-brain axis. Liver and brain samples (4 controls, 6 septic) from a septic mouse model were analysed with both RP-MS and HILIC-MS untargeted approaches. A panel of 9 annotated metabolites, including previously discussed LysoPC (16:0), CE (16:0) and CE (18:1), were uncovered through a meta-analysis which employed three machine learning tools. Subsequently, a correlation analysis was performed between this panel of metabolites and general indicators of sepsis. Based on all the statistical tools applied, glycerylphosphorylethanolamine; a lipid depleted in both liver and brain from septic mice (p<0.05); was selected as the most promising candidate biomarker for endothelial dysfunction during sepsis.

Overall, this work has revealed a panel of key metabolites that are all specifically associated with a range of liver related diseases. Through comparing two types of liver donation, DBD and DCD, LysoPC (16:0) and LysoPC (18:0) were found to be potential biomarkers of liver tissue damage. In the meanwhile, adenine and AMP were discovered to be putative biomarkers of allograft function and survival. Furthermore, inflammatory lipids, ceramides, were also studied in DBD and DCD groups, diverse distributions of five chosen ceramides and correlations with liver dysfunction markers were observed. Moving from human studies to mouse experiments, in a cholestasis

model, a panel of 5 lipids, including phosphatidylcholines and cholesterol esters, were proved to have higher predictive ability compared with traditional enzymes. In addition, glycerylphosphorylethanolamine was chosen as a promising biomarker of endothelial dysfunction in a mouse sepsis study involving liver-brain axis. Future work entails validating these findings in independent cohorts and/or clinical studies.

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List of Abbreviations

ALD	Alcoholic liver disease
ALP	Alkaline phosphatase
ALT	Alanine transaminase
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photo ionization
AUC	Area under the curve
BA	Biliary atresia
CA	Cholic acid
CART	Classification and regression tree
CAWG	Chemical Analysis Working Group
CE	Cholesterol esters
CerS	Ceramide synthases
CIT	Cold ischaemia time
CSC	China Scholarship Council
CT	Computerised tomography
DBD	Donation after brain death
DCA	Deoxycholic acid
DCD	Donation after circulatory death
DESI	Desorption electrospray ionization
EAD	Early allograft dysfunction
EI	Electron impact
FADD	Fas-associated death domain
FDR	False discovery rate
GC	Gas chromatography
GGT	Gamma-glutamyl transferase
GSIS	Glucose-stimulated insulin secretion
HCA	Hierarchical clustering analysis
HCV	Hepatitis C virus
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
ICAM	Intercellular adhesion molecules
IGF	Immediate graft function

INR	International normalised ratio
IRI	Ischaemia reperfusion injury
KC	Keratinocyte-derived chemokine
LC	Liquid chromatography
LD	Living donation
MALDI	Matrix-assisted laser desorption ionization
MAP	Mean arterial pressure
MELD	Model for End-stage Liver Disease
MS	Mass spectrometry
MSE	Mean-squared error
MSI	Metabolomics Standards Initiative
MTBE	Methyl tertiary butyl ether
NAFLD	Non-alcoholic fatty liver disease
NIH	National Institute of Health
NMP	Normothermic machine perfusion
NMR	Nuclear magnetic resonance
NP	Normal-phase
ODR	Organ Donor Register
OPLS	Orthogonal Projections to Latent Structures-Discriminant
PA	Phosphatidic acid
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PCA	Principal component analysis
PELD	Paediatric End-stage Liver Disease
PELF	Peritoneal lavage fluid
PICU	Paediatric intensive care units
PLS	Partial least squares
PNF	Primary non- function
PSC	Primary sclerosing cholangitis
PTC	Percutaneous transhepatic cholangiography
QC	Quality control
RF	Random forest
RLR	Regularised, or penalized logistic regression
RN	Reverse phase negative
ROC	Receiver Operating Characteristic

ROS	Reactive oxygen species
RP	Reversed Phase
RSC	Royal Society of Chemistry
RSD	Relative standard deviation
SCI	Society of Chemical Industry
SD	Standard deviation
SIRS	Systemic inflammatory response syndrome
SOFA	Sepsis-related Organ Failure Assessment
SPE	Solid phase extraction
TLR	Toll-like receptors
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
UHPLC	Ultra-high-performance liquid chromatography
UV	Ultra violet
VIP	Variable Importance for the Projection
WHO	World Health Organization
WIT	Warm ischaemia time

Publications

- **Xu J***, Casas-Ferreira AM*, Ma Y, Sen A, Kim M, Proitsi P, Shkodra M, Tena M, Srinivasan P, Heaton N, Jassem W. Lipidomics comparing DCD and DBD liver allografts uncovers lysophospholipids elevated in recipients undergoing early allograft dysfunction. *Scientific reports*. 2015 Dec 4;5:17737.

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Chapter 1 Introduction

1.1 Liver Related Diseases

Liver related diseases, including non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), cirrhosis, hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), alcoholic liver disease (ALD), hepatitis B and C virus (HBV and HCV) infection, sepsis, cholecystitis, cholestasis, liver failure, and drug-induced hepatotoxicity, are major cause of illness and death worldwide, affecting at least 2 million people in the UK alone [1, 2].

Among all liver related conditions, hepatic steatosis, or fatty liver, is common in the general population [3]. This condition is associated with metabolic syndrome, which is characterized by obesity and/or insulin resistance. Exposure to other risk factors, particularly alcohol abuse and HCV infection, can also cause steatosis [4]. NAFLD is defined as the presence of excessive accumulation of triglycerides in the liver of individuals with minimal or no alcohol intake, comprising a variety of diseases, ranging from simple steatosis to steatohepatitis (NASH), with or without the development of fibrosis and cirrhosis [5]. In addition, viral hepatitis, such as HBV (partly double-stranded DNA virus) and HCV (single-stranded RNA virus), are affecting more than 150 million people as a global health challenge. Persistent infection can lead to progressive liver disease with the development of liver cirrhosis and HCC [6, 7].

In the following chapters, three types of liver associated syndromes, liver transplantation, cholestasis and sepsis, will be reviewed. Interruption of bile flow may have an extrahepatic and obstructive or an intrahepatic and biochemical basis, which causes cholestasis. Unexpectedly, sepsis can also be associated with impaired biliary secretion

[8]. Patients with sepsis often develop a conjugated hyper-bilirubinaemia, suggesting that the secretion of bilirubin into bile is compromised. Moreover, the initiation of hepatic injury in septic conditions usually results from decreased hepatic arterial blood flow [9]. This can lead to poor hepatic microcirculation, resulting in acute cellular and mitochondrial injury, leading to elevated hepatic transaminases, severe hypoglycaemia and reduced production of coagulation factors [9]. Liver transplantation is required when liver failure appears due to severe liver injuries and end-stage liver diseases.

1.2 Liver Transplantation

The liver is a vital organ for human beings with various functions including detoxification and production. Furthermore, it is also the only human internal organ capable of natural regeneration of lost tissue; as little as 25% of a liver can regenerate into a whole liver [10]. Liver transplantation is performed when the liver has been damaged as it loses its normal functional ability. Liver failure can be caused by acute and chronic diseases (as seen in **Figure 1.1**). For acute disease, there are several types, like drug-induced toxicity, viral hepatitis, acute budd-chiari syndrome and metabolic diseases [11]. Chronic liver diseases include alcoholic-related liver disease (ALD), chronic hepatitis B or C, malignancy, non-alcoholic steatohepatitis, autoimmune hepatitis, and other causes of cirrhosis [11].

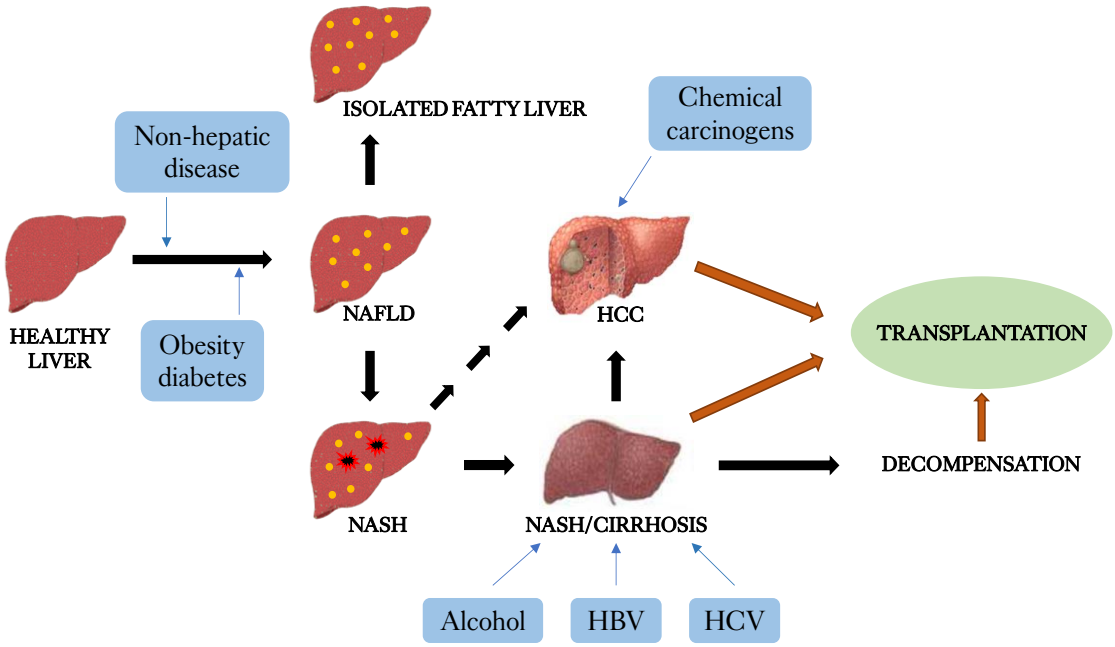


Figure 1.1. Schematic flow shows the development of liver diseases from a healthy liver with various influencing factors (blue boxes). NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus. Figure modified from [2].

1.2.1 Donor Types

Nowadays, the most common types of liver transplantation are donation after brain death (DBD), donation after circulatory of death (DCD) and more rarely living donation (LD). Brain death is a state of cessation of cerebral function, which is considered irreversible. A ventilator which allows the blood to continue to circulate throughout the body is normally provided to a brain-dead donor. Because of this, the organs remain suitable for organ donation [12]. DCD, previously referred to as donation after cardiac death or non-heartbeating organ donation, refers to the donor who does not meet the criteria for brain death but in whom cardiac standstill or cessation of cardiac function occurred before the organs were procured [13]. Living donation transplant is a surgical procedure to procure an organ or portion of an organ from a living person and place it in another person whose organ is no longer functioning properly. Shortage of donors for hepatic transplantation has led to the reintroduction in the last decade of DCD, with the hope of increasing the number of viable grafts for transplantation by 10-20% [14-16]. DBD has been the dominant source of grafts since the introduction of brain stem death criteria (which defines brain death as unresponsiveness and lack of receptivity, the absence of movement and breathing, the absence of brain-stem reflexes, and coma with known cause) in 1968, due to improved graft quality and the potential for multi-organ donation [14, 17, 18].

To safeguard patients, pre-transplant donor screening is used to determine the plausible success rate of liver transplants. The optimal/sub-optimal criteria for liver donors includes age (<50 years/>50 years), weight (<100kg/>100kg), intensive care stay (<5 days/>5 days), functional warm ischaemic time (<20 mins/>20mins, <30mins), cold ischaemia time (<8 hours/>8 hours, <12 hours) and steatosis (<10%/>15%) [19].

According to analysis conducted by the Liver Transplant Program team in King's College Hospital London, 4,632 donation offers were received in their unit from 2001 to 2010. Of 1,579 DCD offers, 621 (39.3%) were accepted for further evaluation and 958 declined. Of the accepted offers, 400 (64.4%) had experienced circulatory arrest after withdrawal of support and their livers were recovered and assessed for graft viability. Of these, 183 (45.8%) grafts were transplanted [14].

In addition, based on the typical donor recovery procedure for DCD, the majority of potential offers are turned down as unsuitable [16]; graft utilization has decreased to 68% with the use of DCD, compared with 90.3% for DBD [14]. The number of livers recovered at DCD but not applied has increased due to four main reasons: DCD long-term outcomes, cholangiopathy, organ quality and related graft dysfunction [20].

DBD donations are widely adopted for liver transplantation, and it is thought to be a better organ source compared with DCD donation since there is no preceding circulatory arrest. However, brain death generates an inflammatory response with the release of various pro-inflammatory mediators, leading to upregulated expression of adhesion molecules on vascular endothelium and subsequent leukocyte tissue infiltration [21, 22]. The pathophysiology of donor liver grafts sourced from circulatory death is evidently different from that of liver grafts sourced from DBD. DCD livers are exposed to a period of warm ischaemia, which is understood to be involved in continued energy consumption by viable cells at body temperature. Though a circulation exists, the organ is still exposed to a hypoxic insult from poor end organ perfusion, which leads to cellular damage [23]. This inadequate level of oxygenated blood through tissue perfusion results in anaerobic metabolism and contributes to increased incidences of

primary graft failure in DCD grafts [24]. The difference in ischemic injury that DCD livers experience in comparison to DBD livers from the time of procurement to donation is illustrated in **Figure 1.2**.

Traditionally, donor organs are kept in an ice box during transportation. Many potential donor organs cannot be utilised because sub-optimal livers do not tolerate conventional cold storage and it is challenging to assess organ viability preoperatively [25]. In recent years, studies and trials on the application of ex-vivo normothermic machine perfusion (NMP) has gained increasing attention worldwide. In practice, following removal from the donor, the liver was attached to the NMP device, where it was perfused throughout the duration of preservation, until it was removed from the device for implantation [26]. Normothermic machine perfusion maintains the liver in a physiological state, averts cooling and allows recovery and functional testing when compared with static cold storage in human liver transplantation [26].

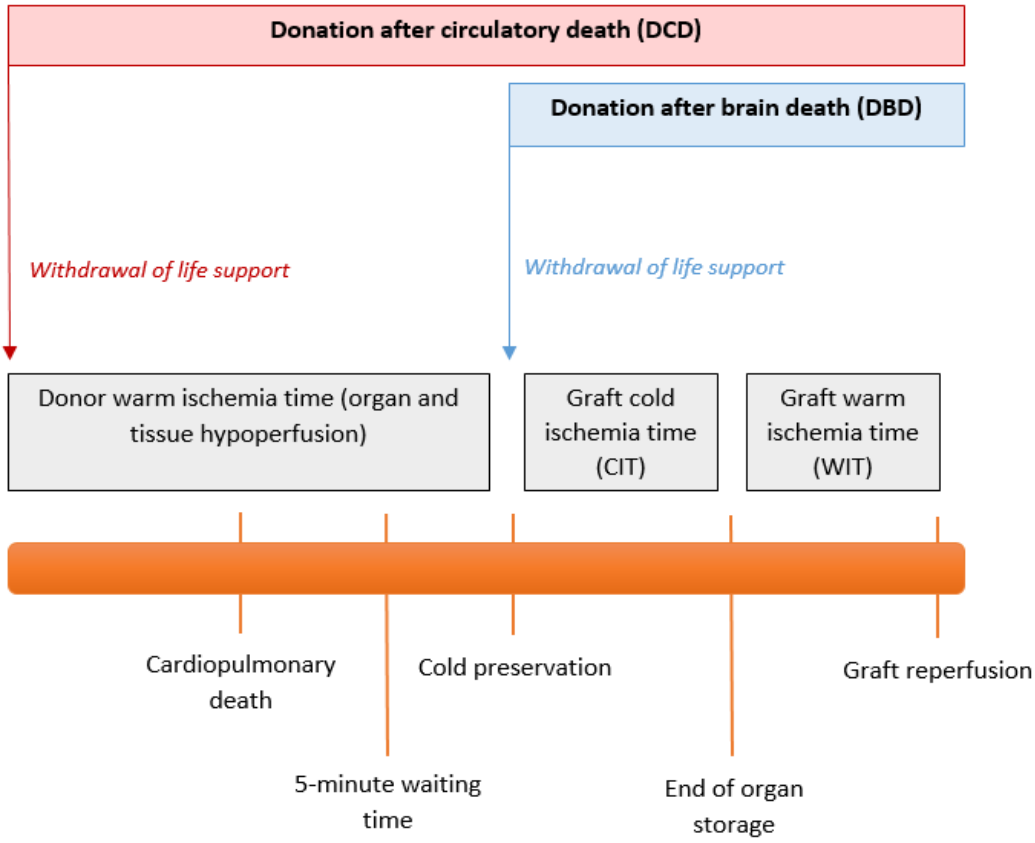


Figure 1.2. The phases of ischemic injuries on liver allografts from procurement to donation after cardiac death (DCD) versus donation after brain death (DBD). Figure adapted and modified from [27].

Since the first successful living donor liver transplantation in 1989 [28, 29], LD has been accepted in most countries in order to decrease the mortality on the transplantation waiting list [30]. Although the ischaemia time involved in LD is the shortest compared with most deceased donation, the risk of the surgery for donors should not been overlooked [31, 32]. Moreover, components of the transplant evaluation process may complicate the donation pairing process. It includes the requirements of donors to have matched blood type to help improve the chances that the donor organ will not be rejected, also, diagnostic tests, *eg*. X-rays, ultrasound procedures and liver biopsy

examination, may be performed to assess the donor's liver as well as the donor's overall health status.

1.2.2 Organ Allocation

Rational and unbiased allocation criteria were not an issue when the first liver transplantation was performed by Thomas Starzl in 1963 [33]. Transplants were restricted to candidate recipients who were lucky enough to be managed at the same institution as the deceased donor, as organs had a very short viability period [34].

Later on, with the acceptance of brain death criteria, donor organ preservation techniques and quality were improved, making the allocation of donated livers at other sites feasible [35]. At the same time, the increasing demand of transplant organs has inflated the waiting lines, this made necessary the development of an impartial and structured organ distribution system [34, 36]. The listing system improved from basing solely on waiting time to disease severity, and to Child-Turcotte-Pugh score after a decade's progress from 1990s [37]. Lastly, the Model for End-stage Liver Disease (MELD) was adopted in the United States in 2002, and a similar scoring system, Paediatric End-stage Liver Disease (PELD), was created for paediatric patients [34, 35].

In the United Kingdom, a different system called UKELD score was developed and validated with 1000 patients on the waiting list, which includes sodium in addition to the MELD score factors (bilirubin, INR of prothrombin time and serum creatinine) [38]. Since hyponatremia is related to neurologic dysfunction, higher risk for development of the hepatorenal syndrome, and death from liver disease, the evaluation of serum sodium

was conducted and has been shown to improve the predictive accuracy of the MELD score [39].

1.2.3 Ischaemia/Reperfusion Injury

Liver ischaemia/reperfusion (I/R) injury is the injury caused by the ischaemia and reperfusion stages in liver retrieval, transporting and implanting. Liver retrieval involves the deprivation of blood and oxygen to the liver, and the livers are cold preserved until the time of transplantation with the aim of keeping them viable [40]. Low temperature protects tissue by slowing the rate of cell death, while the preservation solutions assists to prolong storage time [41].

The damage is sustained during the cold preservation after liver retrieval and during warm reperfusion at implantation in recipients. The reintroduction of host blood supply after reperfusion induces the release of active and potentially toxic chemical products that can cause damage to organs [42]. Furthermore, reperfusion of allografts involves the interaction of host lymphoid cells with the donor innate immune cells, which may affect the immune response after transplantation [43].

In addition, in DCD cases, there is extra warm ischaemia injury before the hepatic cold perfusion [44]. It is also considered as the main reason of primary graft dysfunction or initial function failure after transplantation, both of which can eventually lead to acute and/or chronic rejection [45].

In ischaemia injury, endothelial cells and hepatocytes are damaged in the process of warm ischaemia while cold storage causes injury to sinusoidal endothelial cells [46, 47].

The absence of oxygen during the ischaemic phase leads to interruption of the mitochondrial electron transport chain, debacle of adenosine triphosphate production, and disturbance of electrolyte homeostasis [48, 49]. For reperfusion injury, the formation of reactive oxygen species during the re-oxygenation process stimulates oxidative stress and mitochondrial permeability transition (MPT) [50]. The activation of Kupfer cells and formation of pro-inflammatory cytokines including tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and chemokines lead to neutrophil-related injury, followed by necrosis [51]. As a result of ischaemia/reperfusion insult, necrosis can cause subsequent organ failure [52].

For allografts from those three main donation categories, there are different extents of I/R injury. LD livers are supposed to experience minimal damage compared with allografts from the other two types [53]. Due to extra warm ischaemia in DCD and long ITU stays for DBD donors, the injury including oxidative stress, inflammation and cell death could affect the liver quality and transplantation outcomes to some extent [54].

A primary goal of the pre-transplantation donor evaluation is to determine whether the donor liver is more susceptible to I/R injury and consequently, whether its use will result in graft dysfunction following transplantation [55, 56]. While the assessment is an important patient safeguard, it may also result in otherwise transplantable organs being discarded. Increasing the pool of available and transplantable livers by identifying new pre-transplantation markers of liver quality is thus a high priority need [57, 58].

1.2.3.1 Oxidative stress

Free radicals are highly reactive and consequently short-lived molecules formed in cells as a response to many exogenous and endogenous factors affecting cellular metabolism [59]. The most common ones are oxygen-derived free radicals – hydroxyl radical (*OH), superoxide radical ($*O_2$), nitric oxide radical (NO*), and the nonradicals hydrogen peroxide (H_2O_2), referred as reactive oxygen species (ROS) [60]. ROS, easily reacts with other molecules in a cell, are generated during various physiological and pathological processes [61]. Many diseases are associated with damage from ROS as a consequence of an imbalance between radical-generating and radical-scavenging systems - a condition called oxidative stress [62].

Sources of oxidative stress in liver transplant recipients include I/R injury, immunosuppressive treatment, graft dysfunction and pre-transplant conditions [63]. ROS are signalling molecules at low concentrations, however, at higher concentrations they can harm cellular macromolecules. To prevent this, excessive amount of ROS is eliminated through effective enzymatic (*eg.* superoxide dismutase and glutathione pathway) and non-enzymatic pathways (*eg.* vitamin C, vitamin E and uric acid) [64]. In addition, when the defence mechanisms operated by antioxidants are not sufficient, released ROS triggers several incidents in hepatocytes, inclusive of accretion of $Ca2^+$, production of NO, caspase activation, cytokine gene upregulation, and lipid, protein, and DNA damage [65]. If the rate of ROS production exceeds the capacity of converting them to chemically inert molecules, it results in oxidative injury to tissue [66]. Among the most mentioned modes of injury, lipid peroxidation is thought to be the main cause of hepatocellular damage [62].

Although it is impractical to measure free radicals due to their fleeting life time, oxidative stress can be assessed by other markers. Lipid peroxidation may be evaluated with malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS), exhaled alkanes and plasma oxidizability [67-71]. Determination of oxidative damage to proteins is essentially based on measurement of plasma carbonyl, a marker of protein degradation [72]. The assessment of DNA damage and antioxidant capacity can also be achieved by other tests [73].

1.2.3.2 Inflammatory response

The inflammatory response induced by ischaemia reperfusion is crucial for liver transplantation. A key component of inflammation is the recruitment of inflammatory cells, like neutrophils, lymphocytes, and monocytes to the site of stimulus [74]. At the site of inflammation, tissue damage and augmented oxidative stress were triggered in response to the exposure of chemical mediators, including cytokines and chemokines [75]. Interestingly, in liver inflammation, ROS have been shown to exert their effects on immune cells, leading to up-regulation of gene transcription and modulation of cytokine and chemokine expression [76, 77]. Thus, overexpression of the proinflammatory genes and ROS results in a virtuous cycle, where increased oxidative stress and inflammatory lesion provoke the pathogenesis of I/R injury [78].

Cytokines, like chemokines, interferons, interleukins and lymphokines, which are small proteins that play significant roles in cell signalling, are produced by a wide range of immune cells [79]. To be more specific, lymphokine (cytokines made by lymphocytes),

monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukin (IL, cytokines made by one leukocyte and acting on other leukocytes) are the common types of cytokines [80]. There are both pro-inflammatory and anti-inflammatory cytokines. Widely studied pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 are associated with hepatic tissue damage during I/R injury that may lead to graft dysfunction [81-83].

Except from cytokines, bioactive lipids are also well-known inflammatory mediators[84]. In particular, ceramides, sphingosine 1-phosphate, and ceramide 1-phosphate have been widely implicated in inflammation. The biological functions of ceramides include potently inducing cell cycle arrest and apoptosis [85]. Interestingly, IL-l and TNF, pro-inflammatory cytokines, also activate the sphingomyelin signalling pathway resulting in the generation of ceramides in a spectrum of cell types [86]. Furthermore, ceramide functions as an important signal messenger by exerting its influence on a number of key signal transduction pathways [87, 88]. The distribution and alteration of mostly common ceramides (long fatty acyl chains of 16-28 carbons) in mammals can be explored to have a better view of their functions in liver transplantation.

1.2.3.3 Cell death

Warm and cold hepatic ischaemia followed by reperfusion leads to cell death by apoptosis and necrosis (**Figure 1.3**), which may play a crucial role in reducing organ viability [89, 90].

Apoptotic mechanisms are canonically divided into two main pathways [91]. The first is the 'intrinsic' pathway, or mitochondrial pathway, which is activated by a variety of cytotoxic stimuli including I/R [92]. In hepatocytes following the triggering, proteins such as members of the Bcl2 family are translocated into the outer mitochondrial membrane, thereby enables the release of pro-apoptotic proteins, cytochrome *c*, Smac/Diablo and others, from the intermembrane [49, 93]. Cytochrome *c* forms a complex with apoptosis-activating factor-1, ATP and procaspase 9, where the resultant 'apoptosome' activates the caspase 9 and caspase 3 system [49, 92, 94]. Caspase 3 activity initiates the execution of the final stages of apoptosis, involving cell shrinkage, surface blebbing, internucleosomal DNA hydrolysis, chromatin margination and nuclear lobulation [49, 91].

The second is the 'extrinsic' pathways, that involves the activation of receptors such as the Fas ligand, TNF-α, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors [95]. Activation of these receptors leads to their trimerization, which, in turn, attracts several death domain-containing proteins such as Fas-associated death domain (FADD) and TNF- α receptor-associated death domain (TRADD) to the receptor complex [96]. These death-inducing signalling complexes promote binding of procaspase-8 and its proteolytic activation to catalytic caspase-8. When sufficient caspase-8 is produced, it will activate caspase-3. Caspase-3 acts as the cell's executioner by proteolyzing many cellular proteins [49, 91].

Cell death induced by necrosis differs from apoptotic death in several ways. Single dying cells shrink rather than swell, which happens in apoptosis, whereas big group of cells are found dead in the parenchyma during necrosis [97]. Additionally, necrosis is

characterised by cell swelling with small protrusions named blebs from the plasma membrane [46]. In contrast to the programmed nature of apoptosis, necrosis was considered to occur by uncontrolled processes that led to the 'accidental' death of the cell as a consequence of overwhelming stress [98].

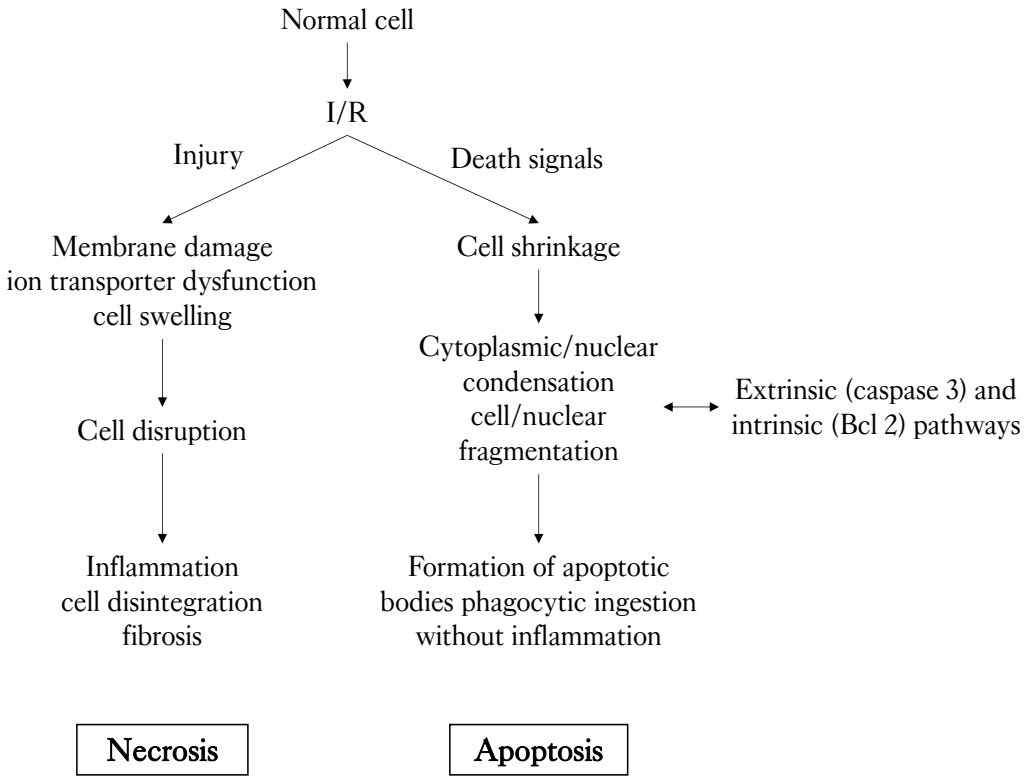


Figure 1.3. Mechanisms of cell death including necrosis and apoptosis in ischaemia/reperfusion (I/R). Figure modified from [89].

Specifically, it is known that cell stress mobilizes and activates a group of serine/threonine kinases named receptor interacting proteins (RIPs) [98]. In particular, RIP1 and RIP3 appear to be functional in coordination as mediators of necrosis [99]. Activation of RIPs 1 and 3, consecutively, results in elevated ROS production either

through activation of NADPH oxidases, or increased mitochondrial oxidant production [100].

1.2.4 Transplant Outcomes

The two main objectives of liver transplantation are to prolong survival and improve quality of life.

At present, patient survival rates at 1 and 5 years have reached 90% and 70%, respectively [101]. Factors that may influence survival rates rely on donor, recipient, perioperative, and postoperative characteristics [102]. Donor parameters that may result in poor outcomes include advanced age, high BMI, long length of hospitalisation, use of vasopressors, and the presence of infection. Recipient parameters include urgent indication, renal dysfunction, and the presence of infection. Perioperative factors comprise cold and warm ischaemia time, blood product requirements, and surgical difficulties. Finally, postoperative factors involve graft dysfunction, renal dysfunction, centre experience, need for mechanical ventilation, and prolonged stay in an intensive care unit [102, 103].

Depending on the severity of graft dysfunction, it ranges from reversible dysfunction (recognised as early allograft dysfunction (EAD)) to an irreversible state of dysfunction known as primary non- function (PNF) [104, 105]. EAD was defined as the presence of one or more of the following defined postoperative laboratory parameters: bilirubin ≥10mg/dL on day 7, international normalised ratio (INR) ≥1.6 on day 7, and alanine or aspartate aminotransferases (ALT/AST) >2000 IU/L within the first 7 days [105, 106].

Furthermore, PNF was described as a graft with poor initial function requiring re-transplantation or leading to death within 7 days after the primary procedure without any detectable origin of graft failure [107, 108]. Apart from EAD and PNF, clinical outcomes can also be classified as immediate graft function (IGF), with values below the cut-off points for EAD [55].

Nowadays, concerns about survival has lessened with improved surgical success, greater attention has been placed on issues that can affect the quality of life among liver transplant survivors [109]. The Short Form-36 Health Status Survey (SF-36) has become the most frequently used quality of life agency in terms of describing and valuing the health status of liver transplant recipients [110]. Specifically, SF-36 contains a 36-item questionnaire covering eight dimensions, including physical functioning, physical role limits, bodily pain, general health, vitality, social functioning, emotional role limits, and mental health [111]. Meanwhile, the 6-minute walk distance (6MWD) is also a widely applied and standardized measure of functional status and exercise capacity for recipients [112]. It measures the distance that survivors are able to walk on a flat, hard surface within a 6-minute period at a self-determined speed without supporting equipment [112, 113].

1.3 Cholestasis

1.3.1 The Prevalence of Cholestasis

A major function of the liver is the production and secretion of bile [114]. Cholestasis is a condition where the bile flow into the intestine is defective [115]. The causes may be intrahepatic or extrahepatic, the latter accounts for 70% of all cholestasis cases [116]. Cholestasis is not a primary cause of death. However, it is the cause of considerable morbidity, since the effects are profound and widespread. Although the principal effects involve the function of the liver and intestine, secondary effects can involve every organ system, leading to systemic illness [117]. Cholestasis can occur not only to both women and men, but also the old, young, and infants [118]. It is reported that women are more affected due to the condition of cholestasis in pregnancy with the incidence rate of 7 per 1,000 pregnancies [119]. On top of this, new-borns and infants are more susceptible and more likely to develop cholestasis as a consequence of immaturity of the liver [120].

1.3.2 The Pathology of Cholestasis

Cholestasis is the result of imbalance between bile acid uptake and efflux. Anomalous hepatic build-up of bile salts provokes disruption of cell membranes and organelles leading to necrosis, inflammation, and fibrosis [121]. The mechanisms of cholestasis can be broadly classified into intrahepatic (also known as hepatocellular), where there are disturbances of bile formation, and extrahepatic (also known as obstructive), where impediment to bile flow occurs after it is produced (**Figure 1.4**) [120]. The typical histopathologic characteristics of intrahepatic cholestasis include the presence of bile

within hepatocytes and canalicular spaces, in association with generalized cholate injury, where the most common causes are drug reactions and inflammatory disorders like cirrhosis and sepsis [114, 122]. Typical of extrahepatic cholestasis is bile plugging of the bile ducts, involving blockage by gallstones, extraluminal compression by a tumor, narrowing of bile duct by biliary atresia, primary sclerosing cholangitis and inflammation of the pancreas [123].

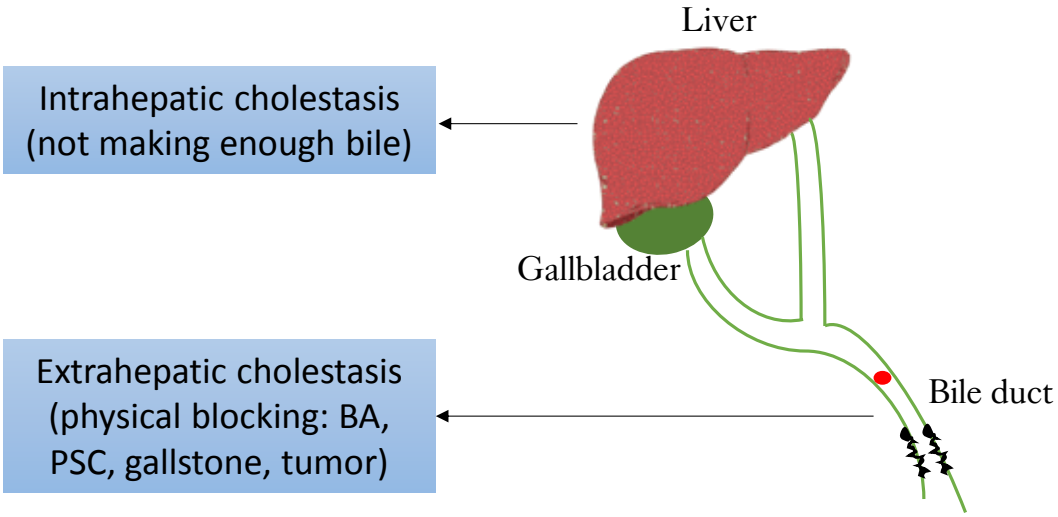


Figure 1.4. The causes of cholestasis are divided into two groups: intrahepatic cholestasis (those originating within the liver) and extrahepatic cholestasis (those originating outside the liver).

Bile is a highly complex water-based medium containing inorganic ions and many classes of organic amphiphiles, the formation of which involves multiple mechanisms [115, 124]. The transport of solute including bile salts, lipids, bilirubin, electrolytes and organic anions into the canaliculus by specific transporters creates chemical and osmotic gradients and promotes water flow by a paracellular pathway [125]. Several of these specific transporters have been identified, and their function has been characterized

(**Table 1.1**). The identification of defective transporters in some familial cholestatic disorders has led to improved understanding of the molecular mechanisms of human cholestasis [126, 127].

Table 1.1. Transporters involved in primary bile formation. Table adapted and modified from [115].

Transporter trivial names(s)	Gene code	Substrate(s)	Defective in
FIC1	ATP8B1	?	PFIC type 1
BSEP(sPgp)	ABCB11	bile salts	PFIC type 2
MDR3 Pgp (in mouse: Mdr2 Pgp)	ABCB4	phosphatidylcholine	PFIC type 3
MDR1 Pgp (in mouse: Mdr1a and 1b Pgp)	ABCB1	amphipathic drugs (neutral and cationic	?
MRP2 (cMOAT)	ABCC2	amphipathic drugs (anionic and neutral)	Dubin Johnson syndrome
BCRP (MXR, ABCP)	ABCG2	amphipathic drugs	?
ABCG5	ABCG5	(phytosterols)	Sitosterolaemia
ABCG8	ABCG8	no direct evidence yet	

Retained bile salts down-regulate new bile synthesis, which results in a reduction of the bile salt pool and reduced enterohepatic recirculation [128]. Furthermore, they induce injury to biological membranes throughout the body, where the liver is the most affected [129]. To be more specific, the retention of hydrophobic bile salts leads to their invasion into membranes, which alters membrane fluidity and function [121]. Bile salt injury of hepatocyte membranes is a key amplifier of cholestasis, while the retention of secondary cholestatic bile acids, such as lithocholic acid, can cause further membrane injury [130].

1.3.3 Clinical Diagnosis for Cholestasis

For the diagnosis of cholestasis, the first approach is always the clinical history, which often is different in both acute and chronic cholestasis [120]. Jaundice is confined predominantly to those with acute cholestasis, which arises as a consequence of elevated serum conjugated bilirubin [122]. A thorough drug history is imperative. Any medications taken within 6 weeks of presentation may be incriminated and only one dose may be sufficient to initiate disease. Having investigated the personal history, the family history also is very pertinent, especially in the case of familiar intrahepatic cholestasis [131]. On top of this, pale stools and dark urine are common signs of cholestasis. Pale stools occur as no bilirubin reaches the gastrointestinal tract and dark urine results from reflux of conjugated bilirubin into blood which is excreted in the urine [132]. The most obvious physical signs of cholestasis are scratch marks and shiny nails secondary to persistent scratching. It is proposed that the increased levels of bile salt which accumulate under the skin causes the itch, while the prolonged rubbing and repetitive scratching make the nails shiny [133]. Other features such as abdominal pain and fever can also appear to cholestatic patients [134].

Further investigation of cholestasis can be carried out when some symptoms mentioned above were present. Typically, the biochemical markers of chronic cholestasis are elevated levels of the bile duct enzymes like ALP and/or GGT [135]. Although the serum ALP level may be increased in bone disease, gastrointestinal disease, or during pregnancy, the GGT level is almost specific to the liver [132]. Increased serum levels of these enzymes are caused by the damaging effect of high concentrations of bile acids on intracellular and biliary membranes [136]. The serum concentrations of conjugated

bilirubin and bile salts are commonly measured parameters as well. As these are retained to different extents in various cholestatic disorders, their relative levels are taken as reference in assisting diagnosis [116]. On top of this, serum transaminases will also be raised in obstructive cholestasis and, to a lesser extent, hepatocellular disease [137].

Apart from the laboratory tests, initial imaging should include ultrasonography which revolutionised the diagnostic work-up of apparent cholestasis because it could clearly distinguish intrahepatic from extrahepatic biliary tract disease [138]. If no dilated ducts are seen on ultrasound scan, endoscopic retrograde cholangiography (ERCP), the gold standard for visualizing the extrahepatic biliary system while providing insight into the cause of the obstruction, can be applied [139]. Besides, percutaneous transhepatic cholangiography (PTC) is normally adopted when dilated ducts are observed on ultrasound scan [132]. The other imaging technique named magnetic resonance cholangiopancreatography (MRCP) is non-invasive which can deliver information about the hepatobiliary and pancreatic systems [140]. Moreover, the less technician-dependent computerised tomography (CT) scan may provide some practical information and can complement ultrasonography, particularly in obese individuals where the latter may be challenging [141]. Lastly, liver biopsy is the single most useful test but it is invasive and the above discussed approaches can be sought before a biopsy is considered [120].

1.3.4 Consequences of Cholestasis and Treatments

The most prominent feature of cholestasis is pruritus [115]. The first line of treatment is the anion-exchange resin, the best-known being cholestyramine. This agent often gives rise to gastrointestinal disorder and is poorly tolerated by some but is effective in 80% [120]. The second line of therapy is the antibiotic rifampin which is effective in 50% [142, 143]. As a third-line therapy, opioid antagonists, is very effective but may lead to chronic pain syndrome when used regularly [144].

Hypercholemia, or increased serum bile salt concentration, is a universal consequence of cholestasis. Hyperlipidemia where serum cholesterol is elevated due to the impaired metabolic degradation and excretion is characteristic of some but not all cholestatic diseases [145]. These patients have complex dietary needs, especially where the disease is enduring. Whilst the fat intake needs to be diminished, the protein and calorie intake needs to be maintained [134]. Hence, careful attention should be paid to prevent fat-soluble vitamin deficiencies (A, D, E, K). The proper approach to treat both complications in cholestatic liver disease is to treat the liver disease itself [120].

A well-described peripheral complication of chronic cholestasis is metabolic bone disease (osteopenia, osteoporosis and occasionally osteomalacia) [117]. Calcium and vitamin D supplementation are the drugs of choices with the addition of bisphosphonates when necessary [146]. Nonspecific therapies with hydrophilic bile acids, like ursodeoxycholic acid (UDCA) that promote bile flow, make physiologic sense [147]. UDCA contributes to improved liver biochemistries, liver histology, and possibly survival [148]. When all approaches have been tried and the consequences of

chronic cholestasis are unresponsive to treatment and unbearable, liver transplantation is curative [149].

1.4 Sepsis

1.4.1 The Definition and Epidemiology of Sepsis

The word 'sepsis' was initially introduced to depict the diseases as a repercussion of self-intoxication with damaging products derived from the colon [150]. Sepsis is a complex and hard-to-define condition with various interactions with other disorders [151]. The developments in defining the spectrum of sepsis syndromes and studying their impact on human life can be traced back to 4th century B.C. as illustrated in **Figure 1.5** [152]. In 1991, sepsis was described by 'systemic inflammatory response syndrome' (SIRS), which is the mechanism of many acute and chronic diseases caused by non-microbial 'irritating causes', such as autoimmune, metabolic or physical insults [153]. However, this non-specific definition does not adequately depict the nature of sepsis, which is caused by insufficiently controlled bacterial, fungal and viral infections manifested by impairment or collapse of microcirculation [154]. The collapse, also known as septic shock, underlies multiple organ failure and culminating in hypotension that is refractory to resuscitation measures. In 2016, the Third International Consensus made the new definition for sepsis as 'life-threatening organ dysfunction caused by a dysregulated host response to infection', and described septic shock as a subset of sepsis in which particularly serious circulatory, cellular, and metabolic abnormalities are associated with a greater risk of mortality than with sepsis alone [155].

According to studies conducted worldwide, incidence of sepsis in adults at the population level ranged from 22 to 240/100 000; of severe sepsis from 13 to 300/100 000; and of septic shock 11/100 000 [156-158]. Mortality rates depending on the setting and severity of disease can reach up to 30% for sepsis, 50% for severe sepsis

and 80% for septic shock [159-162]. Furthermore, it was reported that the prevalence of paediatric severe sepsis yields 8.2%, according to a screening conducted on 6,925 septic children treated in 128 participating paediatric intensive care units (PICUs) across 26 countries [163].

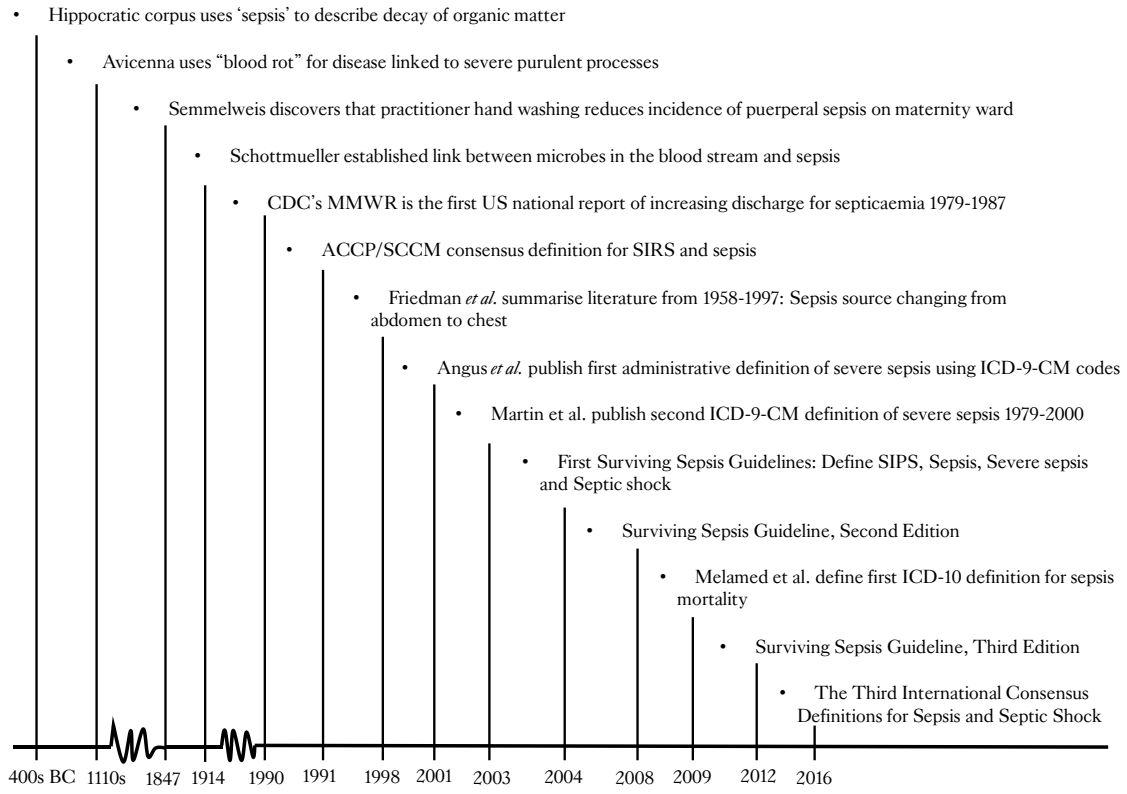


Figure 1.5. Abbreviated Timeline of the Conceptual Definition of Sepsis. Figure adapted and modified from [152].

1.4.2 The Pathology of Sepsis

Extensive research has been done to interpret the pathology of sepsis. Although it turns out to be rather heterogeneous and intricate, the discovery of involved multiple systems facilitates the complete understanding of sepsis [164].

It is widely accepted that the host defence, rather than the microorganisms is responsible primarily for the morbidity and mortality from sepsis [165]. The damage to our own body can be linked with the excessive response of innate immune system. Immune cells identify not only invading molecules (pathogen-associated molecular patterns; PAMPs) but also injured tissues (damage-associated molecular patterns; DAMPs) [166]. From both attacking microorganisms and damaged host tissues, the accumulated DAMPs greatly trigger host immune system, which leads to systemic inflammatory response syndromes (SIRS), also described as 'cytokine storm' [164, 167]. Although the genetic deletion or antagonising some of proinflammatory mediators (*eg.* TNF-α and IL-1) improved the survival of septic animals, clinical trials of anti-inflammatory agents did not demonstrate any benefit [168, 169]. With the acceptance of 'cytokine storm' concept, the understanding of sepsis was further enhanced by the discovery of Toll-like receptors (TLRs) that are pattern recognition receptors (PRRs) [170]. TLRs recognise PAMPs and DAMPs, and stimulate intracellular signalling leading to the production of inflammatory cytokines [166]. While target therapy against one well-known TLR in a clinical trial showed contrary response to high risk and low risk sepsis patients [171], the exploration of other pathophysiological alternation in sepsis is still in progress.

Adequate recruitment of leukocytes to the sites of infection is one of the early and key features of effective immune response [172]. In order to migrate into the sites of infection, leukocytes experience steps of rolling on the blood vessels and adhering onto the vascular endothelial cells [173]. Adhesion molecules such as selectins, integrins, and intercellular adhesion molecules (ICAMs) are among the significant participants [174]. In mild local infection, leukocytes are activated and recruited locally at the site of infection. In septic patients, however, the chemotaxis is impaired and the leukocyte

recruitment to the original sites of infection is rather decreased [175, 176]. Sepsis models tested in majority of knockout mice (various adhesion molecules) revealed worse mortalities when compared to wild type mice [177-179], while enhanced or no reduction neutrophil recruitments were also observed in some cases [180, 181].

Extensive apoptosis of lymphocytes and gastrointestinal epithelial cells was discovered though the autopsy study of patients who died from sepsis [182]. Furthermore, animal and clinical studies suggested that the initial hyperinflammatory response was quickly followed by the development of the sustained anti-inflammatory response [183]. T helper 1 (T_H1) cells and T_H2 cells are a type of T cells that play a crucial role in adaptive immune system where they help the activity of other immune cells by releasing T cell cytokines [184]. During sepsis, the shift from T_H1 response (pro-inflammatory cytokines interferon (IFN)γ, IL-12, and TNF-β production) to T_H2 response (IL-4, IL-5, IL-10, and IL-13 production) occurs, leading into significant immunosuppression. Hotchiss *et al.* proposed that the apoptosis of lymphocytes might further worsen this trend. Notably, the time frame of death from sepsis mostly coincides with the immunosuppressive state, also named immunoparalysis, when immune paralysis occurs due to exaggerated anti-inflammatory response [166, 183]. Immunoparalysis leads to unproductive clearance of septic foci and renders the septic patient more vulnerable to secondary infections, as well as reactivation of latent infections [185].

Surprisingly, the same study revealed that no major cell death was presented in organs like heart, lung, liver and kidney where often demonstrated significant dysfunction in advanced sepsis [182, 186]. In sepsis, the distribution of microvascular flow is altered [187]. Nitric oxide overproduction, antioxidant deficiency, mitochondrial dysfunction,

and diminished adenosine triphosphate (ATP) production were discovered in biopsied muscles of septic patients with organ dysfunction and poor outcomes [188, 189]. However, it was proved that tissue oxygen tension within organs increased [190]. Hence, organ dysfunction in sepsis could be derived from tissues' inability to consume oxygen and subsequent mitochodrial dysfunction [191, 192].

The above section are the known pathologies of sepsis. With the metioned complex mechanisms, identification of more reliable and effective biomarkers is of utmost importance to guide treatments.

1.4.3 Diagnosis and Markers of Sepsis

SIRS criteria (**Figure 1.6**) was applied for the diagnosis of sepsis. When the requisite two or more SIRS conditions were observed in patients with suspected infection, sepsis was confirmed [193]. Yet, with cases where SIRS criteria showed poor discriminant and concurrent weak validity, this approach was considered to be unhelpful [194, 195].

As recommended by the Third International Consensus, for clinical operationalisation, organ dysfunction can be characterised by an increase in the Sequential [Sepsis-related] Organ Failure Assessment (SOFA) score (as seen in **Table 1.2**) of 2 points or more [155]. In out-of-hospital or emergency department, adult patients with suspected infection can be rapidly identified if they have at least 2 of the following clinical criteria that together establish a new bedside clinical score termed quickSOFA (qSOFA) (**Figure 1.6**). Patients with septic shock can be clinically identified by a vasopressor requirement to maintain a mean arterial pressure of 65 mm Hg or greater and serum lactate level greater

than 2 mmol/L (>18 mg/dL) in the absence of hypovolemia. The criteria for sepsis and septic shock were illustrated in **Figure 1.7**.

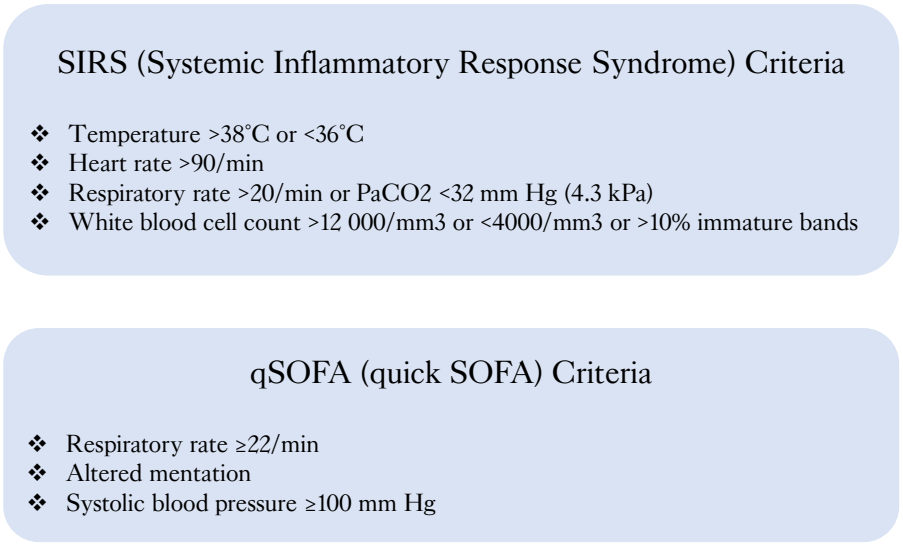


Figure 1.6. Criteria for SIRS and qSOFA. Figure adapted and modified from [155, 193].

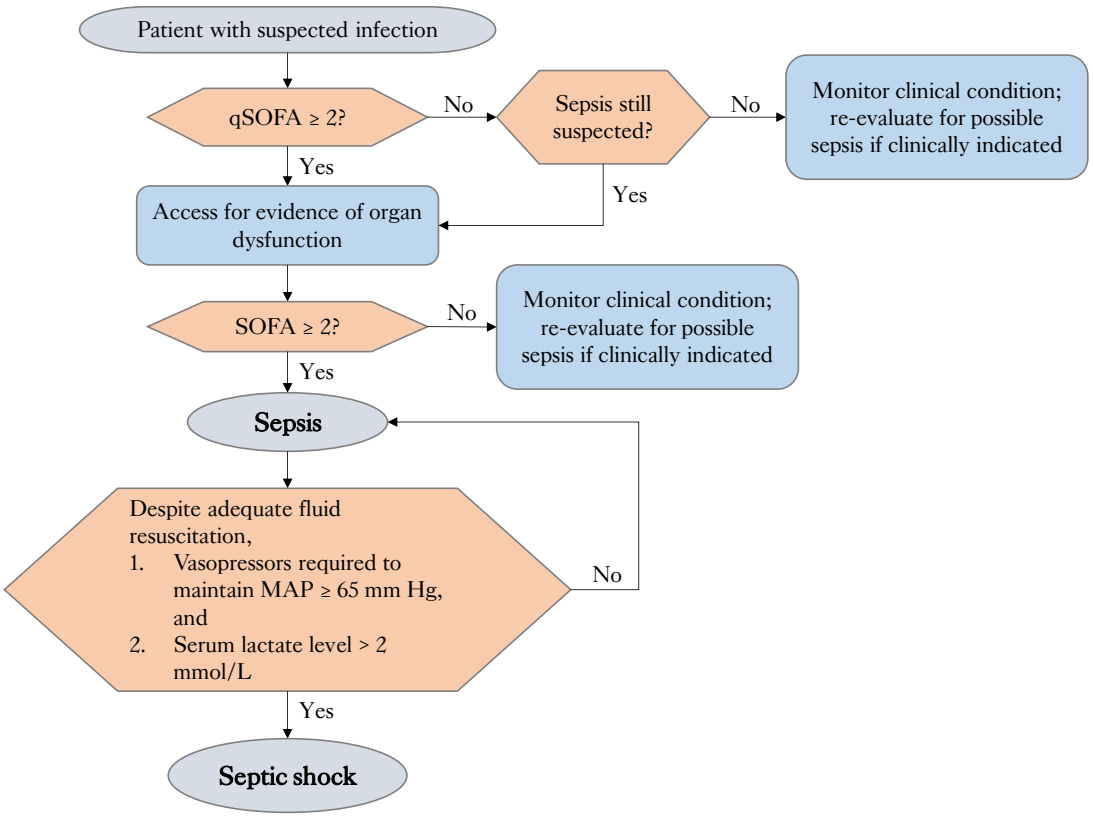


Figure 1.7. Operationalisation of clinical criteria identifying patients with Sepsis and Septic Shock. Figure adapted and modified from [193].

Sepsis is a time-sensitive condition, with delays in either diagnosis or therapy leading to increased mortality [196]. Faster diagnosis of sepsis could potentially reduce mortality, shorten length of stay, and lower hospital costs [197]. Hence, the discovery of new biomarkers as a tool for aiding early diagnosis and rapid appropriate therapies for patients with sepsis can improve patient outcomes greatly [198]. In recent years, more than 150 sepsis biomarkers were reported in a wide range of studies [199]. From those, biomarkers including proteins such as procalcitonin, presepsin, C-creative protein (CPR), CD64, soluble-urokinase-type-plasminogen-activator-receptor (suPAR), soluble triggering receptor expressed on myeloid cells 1 (sTREM-1), IL-6, IL-27, cell-free plasma DNA (cfDNA) and miRNAs have attracted extensive interest [200-202].

Table 1.2. Sequential [Sepsis-related] Organ Failure Assessment Score. Table adapted from [203].

System	Score				
	0	**1**	*2*	**3**	**4**
Respiration					
PaO_2/FIO_2, mm Hg (kPa)	≥400 (53.3)	<400 (53.3)	<300 (40)	<200 (26.7) with respiratory support	<100 (13.3) with respiratory support
Coagulation					
Platelets, $\times 10^3/\mu L$	≥150	<150	<100	<50	<20
Liver					
Bilirubin, mg/dL (μmol/L)	<1.2 (20)	1.2–1.9 (20–32)	2.0–5.9 (33–101)	6.0–11.9 (102–204)	>12.0 (204)
Cardiovascular	MAP ≥70 mm Hg	MAP <70 mm Hg	Dopamine <5 or Dobutamine (any dose)	Dopamine 5.1–15 or epinephrine ≤0.1 or norepinephrine ≤0.1	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1
Central nervous system					
Glasgow Coma Scale score	15	13–14	10–12	6–9	<6
Renal					
Creatinine, mg/dL (μmol/L)	<1.2 (110)	1.2–1.9(110–170)	2.0–3.4 (171–299)	3.5–4.9 (300–440)	>5.0 (440)
Urine output, mL/d				<500	<200

PAO_2, partial pressure of oxygen; FIO_2, fraction of inspired oxygen; MAP, mean arterial pressure.

As a result of the growing appreciation of the important role of immunoparalysis, the focus shifts from inhibiting to stimulating the immune response [204]. Therefore, there is a clear need for suitable biomarkers of a patient's immune status to guide relative treatment. A panel of establishes markers of immunoparalysis were listed in **Figure 1.8**.

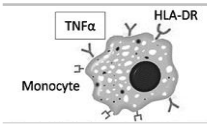
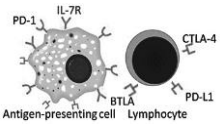
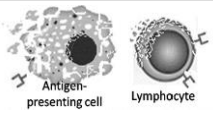
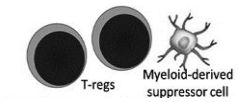
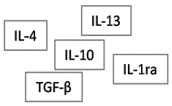
Immunoparalytic mechanisms		Markers
	Monocyte deactivation	↓ mHLA-DR expression ↓ TNFα production upon stimulation
	Tissue macrophage dysfunction	
	Negative regulatory molecules	↑ PD-(L)1 expression ↑ CTLA-4, BTLA expression ↑ LAG-3 and TIM-3 expression
	Downregulation of receptors	↓ IL-7 receptor
	Apoptosis	↑ sFAS, FAS-L ↓ Number of (specific subsets of) lymphocytes
	Suppression of immune cells	↑ CA4+CD25+ (Treg) cells ↑ Myeloid-derived suppressor cells (MDSC)
	Anti-inflammatory cytokines	↑ Concentration of IL-10, IL-13, IL-4, IL-1ra, TGF-β ↑ IL-10/TNFα ratio

Figure 1.8. Mechanisms of immunoparalysis and markers. TNFα, Tumor necrosis factor-α; IL, interleukin; mHLA-DR, Human Leukocyte Antigen on cell surface of monocytes; PD-(L)1, Programmed Death (ligand) 1; CTLA-4, Cytotoxic T-Lymphocyte Antigen 4; BTLA, B- and T-lymphocyte attenuator; LAG-3, Lymphocyte-activation gene 3; TIM-3, T-cell immunoglobulin and mucin protein 3; IL-7R, interleukin-7 receptor; sFAS, soluble Fas; FAS-L, Fas ligand; T-regs, CD4+CD25+ T-regulatory cells; IL-1ra, IL-1 receptor antagonist; TGF-β, transforming growth factor-β. Figure adapted and modified from [204].

Results from previous research indicate that a ratio or panel of markers may have a more robust power in terms of outcome prediction when compared to a single marker, since markers often show mutual correlation [205, 206]. Additionally, when assessing the potential value of markers of sepsis, individual patient characteristics should be taken into account. For example, due to partially shared immunopathogenesis of sepsis and

malignancies, expression of BTLA may be increased in cancer patients regardless of the latent septic condition [185]. Lastly, the kinetics of the established biomarkers need to be considered, since longitudinal monitoring seem to be of more prognostic validity than a single measurement.

1.4.4 Treatments for Sepsis

The prompt treatment for sepsis and septic shock with antibiotics is critical in association with decreased mortality [207]. Broad-spectrum antibiotics (*i.e.* a single gram-positive agent, a single gram-negative agent, a single anti-fungal agent) are normally given first as the identification of a specific type of infection is time consuming [208]. These are designed to work against a wide range of known infectious bacteria and usually cure most common infections. Once a specific bacterium has been recognised, a more focused antibiotic can be used [209]. Moreover, combination therapy, defined as the use of two different classes of antibiotics for a single pathogen, has been broadly discussed [196, 210]. The adoption of a combination of antibiotics can cause resistance and microbiota disruption. However, the utilisation of combination therapy can not only accelerate pathogen clearance, but also assure that pathogen is sensitive to one antibiotic, with regard to significant microbial resistance [196]. The most common combination therapy includes a beta-lactam with an aminoglycoside, fluoroquinolone or macrolide [211].

Besides, the administration of intravenous fluids to improve circulation, perfusion, and oxygen delivery is an essential principle in sepsis management [212]. It is recommended to resuscitate patients in the company of sepsis-induced hypoperfusion with at least

30 ml/kg of crystalloid intravenously within the first 3 hours [213]. Also, medications like vasopressors are used if low blood pressure is inspected, given that oxygen demand goes up for sepsis patients [214].

As the importance of the immunoparalysis during sepsis is increasingly being acknowledged, a few immunostimulatory agents such as granulocyte-macrophage colony stimulating factor (GM-CSF) and interferon γ (IFNγ) that have shown promising results in small studies is currently under investigation in clinical trials [215-217].

1.5 Metabolomics and Its Mechanisms

1.5.1 Metabolomics

Metabolomics is derived from the concept that changes in tissues or bio-fluid can be indicators of diseases [218]. The essence of metabolomics lies in that metabolic status represents the global physiological state in the living organism [219]. Metabolomics involves comprehensive and simultaneous systemic profiling of metabolite levels in a biological system as well as their fluctuations that reflect the response to pathophysiological stimuli, genetic modifications and the surrounding environment [220, 221].

Metabonomics is another terminology often used interchangeably with metabolomics since the analytical and modelling procedures are identical [218]. Metabonomics is defined as 'the quantitative measurement of the multiparametric metabolic responses of living systems to physiological stimuli or genetic modifications' [222]. The emphasis of metabonomics is the metabolic shift over time and interpretation of differences among groups attributable to pathophysiological or genetic stimuli, while metabolomics focus on unbiased analysis of the composition of small molecule metabolites in a given biological tissue or fluid, under a specific set of environmental conditions [222]. However, the divergence between these two terms is philosophical rather than technical.

Metabolomics is an integral part of large scale '-omics' study, and it constitutes 'system biology or medicine' together with genomics, transcriptomics and proteomics (**Figure 1.9**). Genomics is the science of the genomes, initially dedicated to the determination of DNA sequences, has promptly expanded to study the expression profiles and the roles

of both genes and proteins [223]. The leading power behind genomics has been, without a doubt, the Human Genome Project. It began in 1990, and achieved its goal of sequencing the three billion base pairs in the human genome in 2003 [224]. Genome is the total DNA of the studying subject that contains the information for the synthesis of functional proteins [225]. This information needs to be transcribed into mRNA first, following by the synthesis of protein based on the mRNA template. The systemic study of the complete set of mRNAs at a specific timepoint is called transcriptomics [225]. Furthermore, proteomics analyses are the global translated proteins from mRNA and their interactions directing the activities of each cell [226]. Metabolomics is regarded as an extension to transcriptomics and proteomics. It can provide a functional signature of phenotypes for the upstream biochemical information obtained from genes, mRNAs, and proteins as it investigates living organism at metabolite level [227].

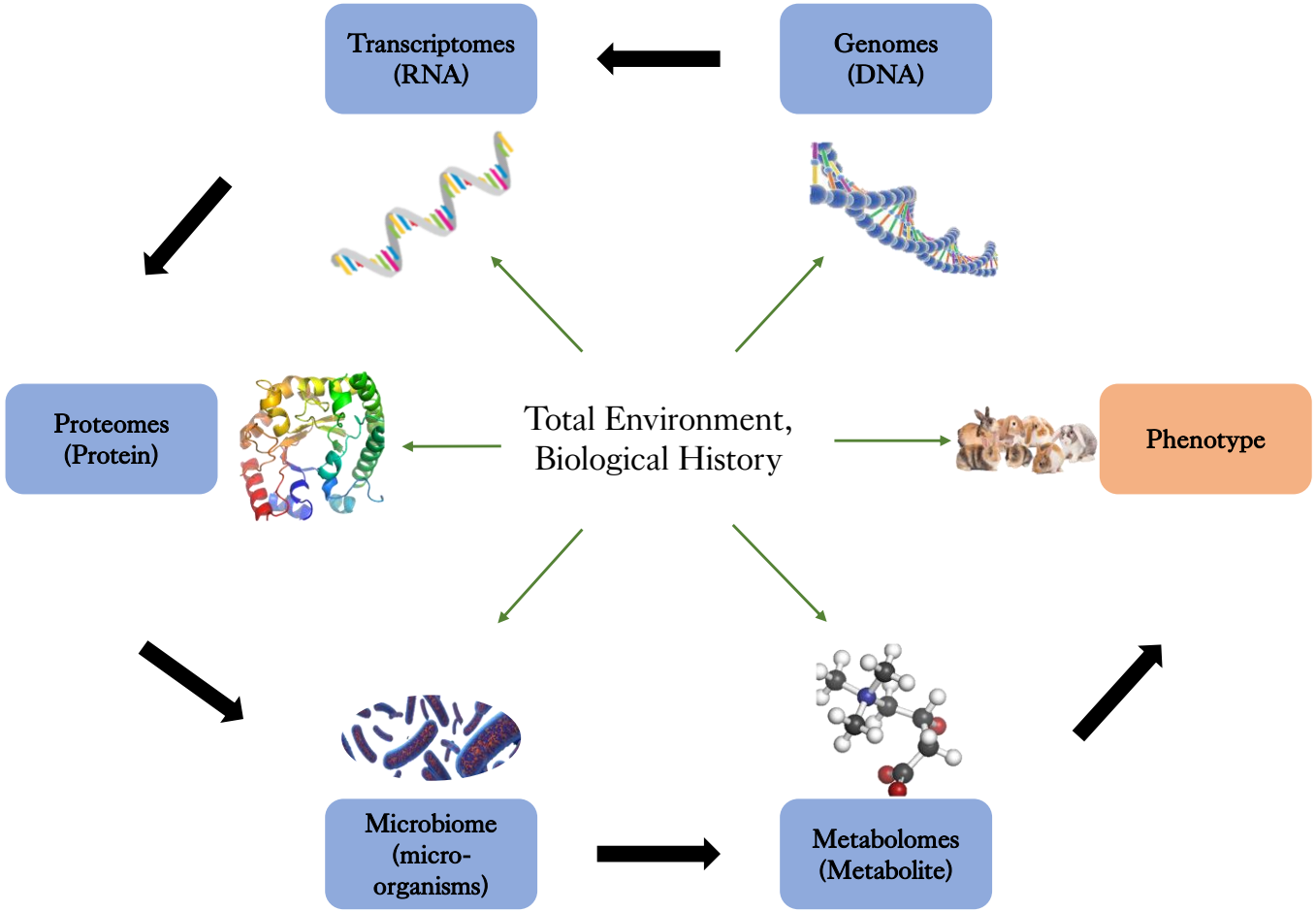


Figure 1.9. A schematic diagram of systems approach to biology. The diagram shows that metabolites are the terminal downstream products of genomes, transcriptomes, proteomes, and microbiome in the whole organisms. DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

Metabolomics, regarded as the apogee of system biology, has a number of inherent advantages compared to other '-omics' techniques [228]. First, since metabolites are the terminal downstream products of the gene expression and protein expression, monitoring the perturbations in a pool of metabolites could reflect underlying disease pathology, disease prognosis and further diagnosis [229, 230]. Next, metabolomics can react rapidly to stimuli or changes, therefore it is capable of presenting the most current view of the system [231]. Moreover, metabolic pathways have been conserved through evolution, which denotes that metabolic pathways are comparable in rodents and humans. Hence metabolic signatures identified in mechanistic and therapeutic studies for animal models can be sometimes translated into human studies [232]. Additionally, the living style and surrounding environment would greatly influence metabolism, which is challenging to unravel their effects from gene-related outcomes. Metabolomics can resolve these issues by monitoring the global consequences of all involving factors regardless of any single contribution to that consequence [218]. Lastly, compared to genomics and proteomics, metabolomics is relatively cost effective and timesaving, and can be applied to various easily accessible biofluids such as serum, plasma, CSF, urine, and peripheral tissues, thus highlighting the clinical utility of this approach [233].

Biomarker is a key terminology in metabolomics field. A biomarker or a biological marker is defined as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic interventions', according to the U.S. national Institute of Health (NIH) Biomarker Definitions Working Group [234]. Another definition suggested by the World Health Organization (WHO) provides a more generalised description. A biomarker is 'almost any measurement reflecting an interaction between

a biological system and a potential hazard, which may be chemical, physical, or biological. The measures response may be functional and physiological, biochemical at the cellular level, or a molecular interaction' [235]. Biomarker is the subgroup of medical signs which can be measured precisely and reproducibly in aiding clinicians to determine disease onset or progression, disease risk factors and the effect of treatments [234, 236]. However, biomarkers may but do not necessarily be applied to assess patients' experience, functions or survivals [237].

Basically, the goal of biomarker discovery is to construct a predictive model that can recognise the difference underlying groups and classify the study objects [238]. To classify samples into specific groups (*e.g.*, healthy *vs*. diseased), high sensitivity and specificity are required for biomarkers, while ease-of-use is also a principal factor [238, 239]. The discovery of novel biomarkers for disease diagnosis and prognosis mainly involves the application of large-scale '-omics' technologies, such as genomics, transcriptomics, proteomics and metabolomics [225]. With the combination of these '-omics' technologies, measuring multiple biomarkers simultaneously to confirm disease onset and progression has captured the attention of clinicians and researchers [240]. Typical biomarkers include body temperature (for fever), blood glucose levels (for diabetes), cholesterol level (for coronary and vascular disease), and BRCA1 mutations (genetic marker for breast and ovarian cancer) [238, 241].

The feasibility of metabolomics for biomarker discovery is supported by the theory that metabolites are important participants in biological systems and that diseases cause disruption of biochemical pathways [242]. The discovery of metabolomic biomarkers can be divided into two approaches, namely, targeted and non-targeted, which will be

discussed later in 1.5.3 with more details. Apart from discovery, there are other major steps along the path of translating a diagnostic biomarker to the clinic, such as pre-validation of biomarkers, analytical development, validation of biomarkers and commercialisation [243].

1.5.2 Analytical Techniques Applied in Metabolomics

The overall size of the metabolome remains elusive in the metabolomics field, but it is estimated to range from tens of thousands to hundreds of thousands of small molecules [244, 245]. Recent development in analytical technologies have allowed measurements of dynamic changes in many molecules from biological sample at the same time.

These technologies include both spectroscopic approaches like ultra violet (UV), infrared (IR), high resolution nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS); and chromatographic approaches, such as liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE), as well as hyphenated techniques, *e.g.*, LC-MS and GC-MS [246]. However, no analytical techniques are available for the analysis of the entire complex nature of the metabolome content. Often a compromise has to be made depending upon a range of criteria such as time frame, cost, properties of the analytes, sensitivity and mass accuracy of instrumentation and the choice of biofluids as different techniques have their own challenges [232]. Generally, multi-parallel techniques are required in metabolic fingerprinting in order to gain a broad metabolic profile. Among all these techniques,

NMR spectroscopy and MS coupled with chromatographic techniques are the main options in metabolomics studies (**Table 1.3**)[247, 248].

1.5.2.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy is a form of absorption spectroscopy. In a magnetic field, based on the absorption of certain nuclei in the molecule and the characteristics of the sample, electromagnetic radiation of different radiofrequencies can be absorbed [249]. NMR spectroscopy in principle works by measuring energy differences between two spin states of certain nuclei when the absorption occurs with the aid of an external magnetic field [250]. The atomic nuclei include ^{1}H, ^{13}C, ^{15}N, ^{19}F and ^{33}P. Amidst them, ^{1}H NMR spectroscopy has been employed widely in metabolomics due to the natural abundance of hydrogen atom in metabolites [251].

As one of the most popular and powerful spectroscopic techniques, NMR spectroscopy is well known for its inherent quantitative precision and high throughput manner [252]. Signal intensity of individual atom in an NMR spectrum is proportional to its molar concentration, enabling direct comparison among all features without the need of calibration curve [253].

Further, the structural elucidation ability of NMR spectroscopy allows rigorous analysis of unknown entities in complex mixtures, such as crude extracts, cell suspensions, intact tissues, or even whole organisms [219, 250]. Metabolite structural identification also allows detection of atomic positions of isotope labels (*e.g.*, ^{2}H, ^{13}C, or ^{15}N) in various isotopic isomer generated during stable isotope tracer studies [254-256]. Thus, detailed

maps of biochemical networks can be profiled by applying NMR spectroscopy [257, 258].

In addition, it requires simple sample preparation and facilitates the high throughput analysis to a large extent [259]. Sample preparation in NMR analysis is minimal with little or no physical/chemical treatment by adding a small amount of buffer and D_2O. With the unprecedented speed and robotic flow-injection method, a standard NMR spectrum can be obtained within a few minutes [260]. Another advantage of NMR spectroscopy is that it is a non-destructive technique [261]. This feature makes it possible for samples to be re-analysed by NMR or be re-used by other analytical platforms, which has great importance in the study of metabolomics [246].

In summary, NMR is an ideal analytical platform for the metabolomic field for universal merits. However, it has a few drawbacks, and the relatively poor sensitivity is a weakness which limits the observation to around hundreds of metabolites, accounting less than 10% of the whole metabolome in an organism [262]. Another limitation is the spectral complexity with overlapping of signals, compromising the clear identification of features [253].

1.5.2.2 Mass Spectrometry (MS)

Mass spectrometry is another mainstay technique in metabolomics research and it has undergone extensive development over the past few decades [246]. In a mass spectrometer, analytes can be detected after selection when they are charged, even with low concentrations. However, in the setting of an NMR spectrometer, energy

differences between two spin states of a nuclei is too weak to be measured and hence requires a much higher sample concentration [263]. Because of its high sensitivity when compared to NMR spectroscopy, MS allows detection and measurement of picomole to femtomole concentrations of numerous primary and secondary metabolites [264]. In addition, MS provides spectral information which contributes to the metabolite identification, either through the measurement of molecular mass with high accuracy or by the collection of fragmentation ions [265].

MS performs the analysis by three steps: transforming analytes into gaseous ions, ion separation according to their mass/charge (m/z) ratio and detecting the ionized analytes [266]. Modern MS offers a variety of ionization and mass analyser technologies with different operational principles and functions [240]. In the metabolomics field, the most common ionisation techniques are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI), with the characteristic of ionizing metabolites of a wide range of polarity (**Figure 1.10**) [267]. These techniques are especially prominent in untargeted analysis due to their 'soft' ionization feature, generating little or no fragmentations which can aid identification of unknown metabolites [267]. Other often applied techniques including matrix-assisted laser desorption ionization (MALDI), desorption ESI mass spectrometry (DESI) , nanostructure-imaging MS (NIMS) and secondary ion MS (SIMS) are imaging mass spectrometry techniques and those can reveal the localization of selected metabolites within a tissue sample [268-272].

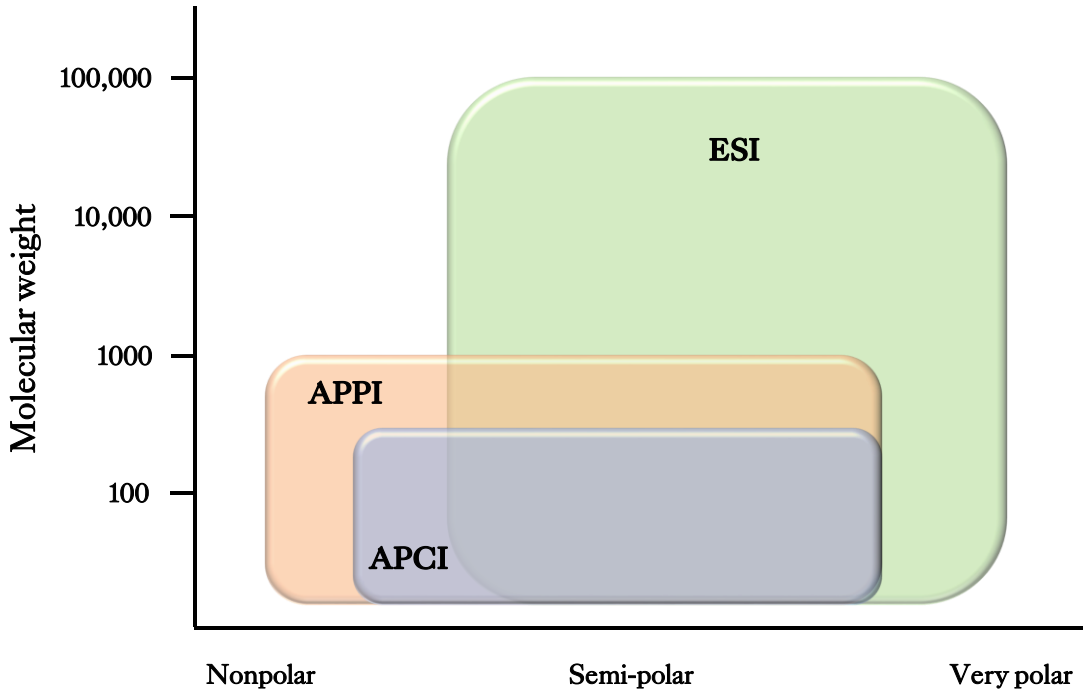


Figure 1.10. Application of soft ionization techniques, ESI, APPI and APCI. ESI, electrospray ionization; APPI, atmospheric pressure photo ionization; APCI, atmospheric pressure chemical ionization. Figure adapted and modified from [267].

Mass analysers can be categorised based on the different resolving powers. High/ Ultrahigh resolution MS include Fourier transform-Ion cyclotron resonance-MS (FT-ICR-MS), orbitrap and time of flight (TOF) [273]. They provide accurate mass measurements and precise metabolite quantification. Low resolution MS such as ion trap and single quadruples are also utilised in metabolomics. Each of the preceding analysers has its own advantages and disadvantages. And the selection of mass analysers, as well as ionization techniques, depends on the aim of the project, throughput and instrumental costs [274]. Mass spectrometer can also be operated in tandem mode (MS/MS) where second stage of MS (MS2) works in sequence, separating and detecting fragmented ions from the first stage of MS (MS1).

As mentioned earlier the main strength of MS is sensitivity. However, it also has limitations. The quantification capability of MS is not as reliable as NMR spectroscopy. To obtain accurate quantification for a specific metabolite, an isotope-labelled standard with known concentration must be added during sample treatment as the internal standard. This strategy can be costly and the availability of labelled endogenous standard is scarce, which makes it especially impractical for untargeted analysis [275]. Another drawback of MS is the biased measurement. MS can only detect metabolites that can be turned into gaseous ions, and in order to increase the detecting coverage of metabolites, biological samples often have to undergo multiple steps of sample preparation [276]. Additionally, multiple components entering the MS at the same time can impact ionization and cause ion suppression skewing the measurement of molecules.

MS can be employed in two modes, standalone (direct infusion MS, DIMS) or coupled with chromatography. DIMS approach is popular in metabolomics domain because it is a time saving and high throughput screening tool. With the availability of advanced MS instruments (FT-ICR-MS and orbitrap) that provide both high mass resolution and high mass accuracy, DIMS is being utilised broadly [277].

Known disadvantages of DIMS, include ion suppression co-elution of metabolites into the mass spectrometer, the production of complex spectra and the limited metabolite identification to putative annotations at best [278, 279]. In order to surmount the challenges that occurred during DIMS analysis, MS is often coupled with chromatographic tools [280].

1.5.2.3 MS coupled with Chromatography

Separation techniques coupled with MS have been extensively utilised in metabolomics. The approach combines the separation power of chromatographic technologies with the high sensitivity and selectivity of MS. Separation prior to detection reduces the matrix effect (where the matrix (*eg.* urine, plasma or serum) coextracted with the analytes can either enhance or weaken the signal response), and ion suppression that enables more accurate identification and quantification of metabolites [281]. Currently, there are three separation techniques incorporated in MS-based metabolomics, namely gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE).

The application of GC-MS in metabolomics can trace back to the 1950s, it was described as the gold standard even though it has its own limitations those will be discussed later [266]. Now it is still one of the most employed platforms for comprehensive analysis of metabolites, especially for hydrophilic molecules [282]. The combined technique provides high sensitivity, reproducibility, and resolution, so that it can be used in trace analysis [283].

Capillary columns with greatly increased separation efficiency when compared with packed column is commonly chosen [264]. In addition, the constant hard ionization, electron impact (EI) ionization energy of 70eV, offers reproducible mass spectra and complete information of compound fragments [284, 285]. These allow more simplified metabolite identification by comparing retention time and standardised mass spectra data to those in public and commercial reference libraries [286-288]. In metabolomics, GC coupled to single quadruple MS is often adopted due to its reliability, effectiveness and affordable cost.

However, only volatile and thermostable compounds can be detected through this method [285]. Non-volatile compounds need to be converted into volatile forms through derivatization prior to analysis [282]. Generally, a two-step derivatization is required, firstly oximation and subsequently silylation [289]. Since anhydrous condition is needed for derivatized reactions, any presence of water would interrupt the process. Also, the drying process with continuous heating may lead to the loss of volatile compounds [266, 290].

Since GC-MS is less adaptable to large and highly polar metabolites due to their poor volatility even after being derivatized. Using other chromatographic tools such as LC can analyse such polar compounds. LC-MS is an important technique in metabolomics in association with its flexibility [291].

Normal-phase (NP) or reversed-phase (RP) columns can be employed for LC analysis, while both positive and negative ionization can be applied for MS to maximize the metabolites coverage [292]. Hydrophilic interaction liquid chromatography (HILIC) which is ideal for highly polar and ionic metabolites has also been widely applied to provide a complementary view of metabolome along with RP-LC [293]. In addition to the diversity of columns, different combinations of mobile phases (isocratic or gradient) further allow tailored separation of metabolites of interest [294]. The appearance of ultra-high-performance liquid chromatography (UHPLC), incorporating with even smaller particle sizes (sub-2μm) in column and a pump with a maximum pressure of 100 MPa, yields significantly improved resolution, sensitivity and even the speed of LC analysis [295].

These advantages have allowed LC-MS to be the dominant analytical platform in metabolomics. Yet, the development of LC-MS was once hampered because of a number of limitations [294]. The major weakness is the inconsistent quantitative precision caused by analytical variation. Various combinations of columns and mobile phases enable LC-MS to be flexible, but also sacrifice analytical variation such as drift of retention times, alteration of intensity values, and occasional modification of m/z values [296]. To assess these analytical variations, quality control (QC) samples are suggested to be repeatedly analysed throughout the entire experiment [297]. QC samples are aliquots of a sample pooled from all analysing samples. By inspecting QC samples along the whole run, the presence and types of variations can be evaluated.

CE-MS is another valuable liquid phase technique because of the alternative separation mechanism involved [296]. CE which separates analytes based on the charge and size is particular suitable for separation of highly polar and ionic molecules, whist neutral metabolites can be separated via employing charged surfactants [298]. CE-MS analysis is fast and efficient with minor sample pre-treatment and minimum organic solvent consumption [280]. Nevertheless, the application of CE-MS is still relatively limited in metabolomics field, mainly because it suffers from poor detection sensitivity and robustness [299].

Furthermore, the innovation of two-dimensional (2D) separation methods has boosted separation efficiency and peak capacity to a significant extent [280]. 2D separation techniques facilitates the analysis of complex biological samples with an unsurpassed resolution power as well as improved sensitivity compared to traditional single-

dimension separation [300]. LC ×LC and GC × GC techniques have been applied in numerous metabolomics studies, demonstrating satisfied separation [301, 302].

Table 1.3. Summarised strengths and limitations of analytical platforms applied in metabolomics studies.

Analytical platforms	Strengths	Limitations
NMR Spectroscopy	Universal detection Precise quantification Simple sample preparation High throughput High reproducibility Structural identification High efficiency	Low sensitivity High instrumentation cost
DIMS	High throughout High sensitivity Fragmentations supported structure determination	Susceptible to ion-suppression Unable to differentiate isomers
GC-MS	Wide range of database High throughput Higher sensitivity compared to NMR	Complex sample preparation Insufficient Structure information compared to NMR and LC-MS
LC-MS	High sensitivity High selectivity Fragmentations supported structure determination Wide range of compounds coverage	Poor reproducibility Less structural information than NMR

NMR, nuclear magnetic resonance; DIMS, direct infusion mass spectrometry; GC-MS, gas chromatography - mass spectrometry; LC-MS, liquid chromatography – mass spectrometry.

1.5.3 Data Treatment Strategies in Metabolomics

Current metabolomics data analysis strategies are usually categorized into non-targeted and targeted approaches for variable selection following data extraction and preprocessing. In the data treatment stage, non-targeted metabolomics approach is normally followed by the targeted approach (**Figure 1.11**).

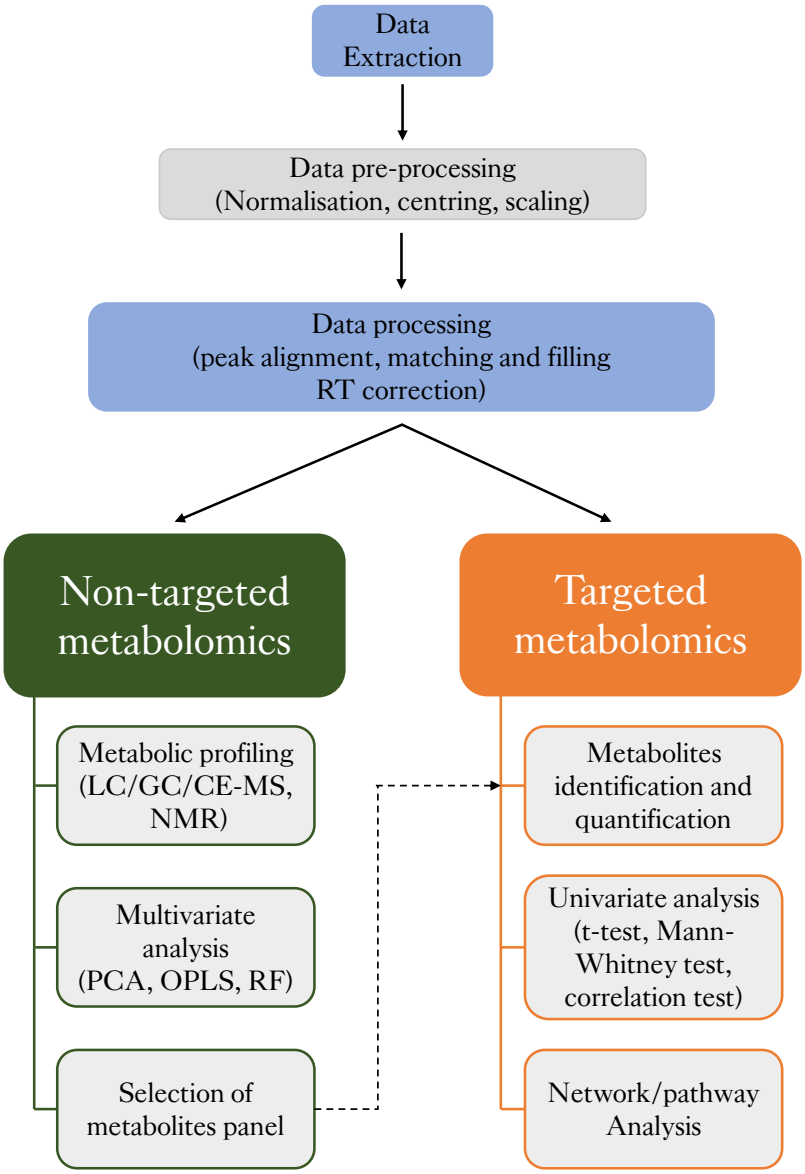


Figure 1.11. A schematic diagram of data treatment workflow in metabolomics. After conducting data extraction, pre-processing and processing, sample cohorts can be subjected to untargeted and targeted metabolomics analyses. Often, non-targeted analysis can also be followed by targeted analysis once relevant metabolites are selected from multivariate analysis.

Untargeted or global metabolomic analysis allows for an assessment of the metabolites extracted from a sample and can reveal novel and unanticipated perturbations [240]. Untargeted analyses are most effective when implemented in a high-resolution mass spectrometer, to facilitate structural characterization of the metabolites. Its primary

advantage is that it offers an unbiased means to examine the relationship between interconnected metabolites from multiple pathways [303]. In the data pre-treatment stage, there are several platforms available for a series of data processing procedures including peak detection, peak alignment and peak matching (*e.g.*, XCMS, MZmine, TagFinder, MarkerLynx, and MetaboAnalyst) [304-307]. Usually, each feature (variable) can be regarded as a dimension, and each sample can be represented by the variables information. Every sample has a unique coordinate in the hyperspace consisting of all variable dimensions [308]. Since the data set of non-targeted metabolomics is extremely large, the application of computational classification tools that will be discussed later can ease the process of extracting the metabolites of interest from a big data matrix.

Targeted metabolomic analyses measure the concentrations of a predefined set of metabolites. Specific metabolites of interest can be directly identified or verified in the databases. This type of analysis can be used to obtain exact concentrations of metabolites identified by untargeted metabolomics, providing analytical validation [309]. The quantitation/semi-quantitation of interested metabolites could be achieved by normalisation with internal standard, external standard and total ion current. Among these methods, the comparison between samples and standards provide more confident and accurate information. A standard curve for a concentration range of the metabolite of interest is often prepared, so that accurate quantification can be gained. For univariate analysis, according to the sample size involved in studies, non-parametric or parametric statistical tests could be applied for metabolites concentration comparison between or among groups, and subsequent clinical outcome correlation is preferred if available [310].

In addition, network modelling and pathway-mapping tools can help us to understand the parts that identified metabolites play in relation to each other and in biological aberrations. Thereafter, metabolites can be placed into context with upstream genes and proteins to lead mechanistic investigations through established and comprehensive metabolic network resources like Kegg [311].

1.5.3.1 Computational classification tools

Metabolomic data analysis becomes increasingly challenging when dealing with clinical samples with diverse demographic and genetic backgrounds and various pathological conditions or treatments. Many classification tools, such as multivariate analysis (MVA), and machine learning algorithm, have been successfully used in metabolomics [312].

MVA is one of the statistical methods which analyse observations (samples) and more than two variables [313]. It has shown high efficiency and reliability for the analysis modelling of complex metabolomics data. MVA conducts dimension reduction while retaining the maximum variability and extracting the metabolites of interest from complicated matrix [238].

Principal component analysis (PCA) is a non-supervised multivariate method and is most widely used in metabolomics study, to visualize the overall trend within the multidimensional dataset [314]. This non-supervised method is used to simply classify groups and check outliers, giving that sample identity is unknown. Moreover, the supervised multivariable analysis techniques, *e.g.*, partial least squares (PLS), PLS-discriminant analysis (PLS-DA), and orthogonal projections to latent structures-

discriminant analysis (OPLS-DA), use the prior information about sample classes, giving better information in biomarkers discovery based on distinguish features related to different groups [315].

However, it has long been known that PLS (and all of its variants, PLS-DA, OPLS, OPLS-DA, *etc.*) can easily generate models that over fit the data, and that over fitting of the model needs to be assessed using cross-validation [312]. Multivariate analysis of metabolic fingerprinting datasets is performed most often with the linear projection-based methods of PCA, PLS and OPLS, but these are by no means the only tools available. Supervised machine learning methods include random forest (RF) and logistic regression are gaining extensive attentions in metabolomics field.

Random forest (RF), is reported as an excellent classifier with the following advantages: simple theory, fast speed, stable and insensitive to noise, little or no overfitting, and automatic compensation mechanism on biased sample numbers of groups [316]. It is a combination of tree-structured predictors (decision trees). Each tree is created via a tree classification algorithm and generates a unit vote for the most popular class based on a bootstrap sampling (random sampling with replacement) of the data. The simplest random forest with random features is formed by selecting randomly, at each node, a small group of input variables to split on. The size of the group is fixed (normally set as the square root of the number of total variables) throughout the process of growing the forest. Each tree is grown by using the CART (classification and regression tree) methodology without pruning [312].

The standard logistic regression model predicts the probabilities of a sample being a member of either of two groups for a set of metabolite peak intensities. However, it has

difficulty handling a large number of variables [317]. Typically, variable selection is performed before fitting a standard logistic regression model. Variable selection is mainly performed to include clinically relevant and statistically significant variables into the model, while excluding noise or redundant variables [318]. Purposeful selection, best subset, stepwise selection and bagging method are common procedures for this purpose [319]. Penalized/regularised logistic regression, a variant of the logistic regression model, can handle a large number of variables and has a built-in stepwise variable selection process [320]. Apart from the previously mentioned supervised machine learning approaches, hierarchical clustering analysis (HCA) and K nearest-neighbour clustering may also be applied to multivariate spectral data to reveal differences between classes without supervision.

The classification performance of classification tools can be evaluated and compared using several approaches: cross-validation, R^2/Q^2 plot, ROC, reduction of variable number and utilising training/test datasets. These are important aspects for a classifier to assess prediction ability and stability, overfitting, diagnosis potential, and variable number dependence.

1.5.3.2 Metabolites identification and databases

Initial putative metabolite identifications can be made based on the accurate m/z of the mass spectral ion (parent ion). This is aided using comprehensive metabolite databases such as METLIN, Human Metabolome Database (HMDB), and MassBank [321-323]. Further identification can then be carried out on the isolated ion, followed by matching with authentic standard or spectral libraries, in order to obtain characteristic fragments

(daughter ions) and retention time information to distinguish the ion from structural isomers [240].

The identification of metabolites in metabolomic samples is challenging as it has to discriminate (i) metabolites of different nominal masses; (ii) metabolites with the same nominal mass but different molecular formulas and monoisotopic masses; and (iii) metabolites with the same nominal and monoisotopic mass, but different chemical structures (including chirality and isomerism) [324]. Therefore, The Chemical Analysis Working Group of the Metabolomics Standards Initiative has reported four levels of metabolite identification confidence (**Table 1.4**) to aid with reporting metabolites identification [325].

Table 1.4. The four levels of metabolite identification confidence. Defined by the Metabolomics Standards Initiative [325].

Level	Confidence of Identity	Evidence
1	Confident identification of compounds	Comparison of two or more independent and orthogonal properties (RT, m/z, fragmentation mass spectrum) with an authentic chemical standard analysed under identical analytical conditions
2	Putative annotation of compounds	Based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries, without reference to authentic chemical standards
3	Putative annotation of compound classes	Based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class
4	Unknown compounds	Although unidentified and unclassified, these metabolites can still be differentiated and quantified based upon spectral data

RT, retention time; m/z, mass/charge ratio.

1.6 Application of Metabolomics in Liver Related Diseases

Metabolomics, or small-molecular metabolic fingerprinting, is an emerging discipline with a wide-range of applications. Metabolomics has been applied together with the pattern recognition and multivariate statistical analysis on complex datasets [233, 326]. Studies based on metabolomics have been expanding to apply in a variety of fields: *e.*g. Alzheimer's disease, anti-doping, gut microbial activities, and liver related diseases [327-329].

For the application of metabolomics in liver transplantation, studies about early allograft dysfunction and potential biomarker of DBD transplant type have been conducted [55]. There is also research performed in tissue metabolomics of hepatocellular carcinoma (HCC), revealing that monounsaturated palmitic acid can act as a marker for HCC progression and poorer patient survival [330]. Additionally, Gonzalez *et al.* found increased serum levels of several metabolites, including those also discussed in this thesis, LysoPC (18:0) and PE (34:2), were significantly modified due to acute liver injury while showing a high correlation with the degree of liver damage determined by histological examination of the livers [331].

The therapeutic effect of herbal medicine dose on cholestasis was investigated using metabolomics coupled with multivariate data and pathway analysis. Several bile acids and amino acids were found to be associated with the dose influence [332]. A study conducted by Aoki *et al.* observed that antioxidative and cytoprotective metabolite levels were markedly increased in urine of cholestatic rats, while the concentrations of many bile acids were also elevated in plasma and urine samples [333]. Furthermore, an

NMR metabolomic study has been performed in cholestatic rats in an attempt to use urinary biomarkers to distinguish the two mechanisms (extrahepatic and intrahepatic) [334].

In the field of sepsis, a global metabolomic profile in plasma broadly differed between survivors and non-survivors. From 423 identified small molecules, 17 % of them showed significant differences between two groups. In the meanwhile, broad differences were also present in pathways of oxidative stress, bile acid metabolism, and stress response [335]. Izquierdo-García *et al.* were able to build a predictive model discriminating between septic and non-septic rats. The discriminant metabolites identified in lung tissue and serum include alanine, creatine, phosphatidylethanolamine, and myo-inositol [336].

Over the past decades, metabolomics has been utilised in the field of hepatology. There is no other organ like liver where such a plethora of both hydrophilic and lipophilic metabolites are metabolically interchanged [2]. The rates of metabolism and energy production and consumption found in the liver are speedy and non-comparable to other organs [337]. The hepatic metabolome is therefore a highly complex and dynamic flux of small metabolites. Overall, an extensive number of studies have applied metabolomics in order to identify 1) metabolites which would potentially be used as biomarkers for liver related diseases and 2) perturbations in metabolic pathways that reflect variations associated with disease pathology. Metabolomics has manifested its capacity of being an innovative platform for the analysis of the liver related diseases.

1.7 Aims

The liver is the source of a myriad of endogenous metabolites and precursors used by other organs, it also houses abundant detoxication enzymes that play crucial roles in an array of diseases. The previous section reviewed liver transplantation, cholestasis, sepsis and metabolomics. It highlighted metabolomics as an approach to further understand the biochemistry of liver related diseases and to facilitate the discovery of potential endogenous biomarkers.

In this thesis, untargeted lipidomics with RP-MS technique will be employed for liver tissues from two different liver transplant donation groups, DBD and DCD, to compare the lipid fingerprints and determine whether differences in metabolite levels reflect tissue damage. Targeted lipidomics focusing on pro-inflammatory mediators, ceramides, will also be conducted to assist the understanding of IRI during liver transplantation. To extend the discovery from lipid studies, polar metabolites using an additional HILIC-MS metabolomics method was applied in an untargeted discovery approach.

Then both plasma and liver samples of mice from a cholestatic model were studied. Bile acid and lipid levels were measured using RP-MS methods to find lipid signatures associated with cholestasis.

Lastly, RP-MS and HILIC-MS will be combined to investigate the full metabolic profile of liver and brain biopsies from mice in a septic model. Three machine learning techniques were applied to facilitate the seeking of sepsis associated metabolites.

Chapter 2 Lipidomics comparing DCD and DBD liver allografts uncovers lysophospholipids elevated in recipients undergoing early allograft dysfunction

The named researchers below contributed to this chapter by conducting the following experiments.

1. Mr Wayel Jassem (King's College Hospital, UK): Biopsies collection and clinical data collection.
2. Mr Parthi Srinivasan (King's College Hospital, UK): Biopsies collection.
3. Mr Nigel Heaton (King's College Hospital, UK): Biopsies collection.

2.1 Introduction

Liver transplantation is the most viable solution to a range of acute and chronic end-stage liver diseases [338]. The global prevalence of liver disorders such as cirrhosis, hepatitis B and C and non-alcoholic fatty liver disease have resulted in a marked increase in the demand for transplantation [339-342]. However, widespread shortfalls in donor organ availability mean that the demand for transplantation greatly exceeds its actual occurrence [338]. This crippling donor shortage has led to the increase use of organs from 'marginal' donors [343, 344], including those obtained from donation after circulatory death (DCD). The use of DCD livers remains limited as organs are exposed to a significant period of warm ischaemia prior to retrieval and have poorer patient outcomes [345-347]. It is estimated that up to a fifth of donation after brain death (DBD) organs do not meet the strict clinical criteria for transplantation, and are thus discarded [348], compared with even higher losses reported for DCD grafts [16]. A primary goal of the pre-transplantation donor evaluation is determining whether the donor liver is more susceptible to graft dysfunction following transplantation [55, 56]. While the assessment is an important patient safeguard, it may also result in otherwise transplantable organs being discarded. Increasing the pool of available and transplantable livers by identifying specific pre-transplantation markers of liver damage is thus a high priority [57, 58].

Elevated liver-enzyme levels are widely accepted as the standard for liver injury, however these tests lack specificity as they can be affected by medication and other syndromes [349]. Biomarker discovery in the context of liver pathophysiology has been predominantly genomic and transcriptomic-based [350]. In order to find metabolite

markers in liver tissue, targeted and metabolite phenotyping strategies have been applied to find markers relevant to liver transplantation [351, 352], findings highlighted lipid associations to early allograft dysfunction (EAD) and recipient clinical outcomes [55, 353-355].

Due to different mechanisms implicated in brain death and circulatory death, as well as distinct retrieval procedures involved, it is important to obtain comprehensive interpretation of changes at metabolites level in both donation groups. Hence, the aim of this chapter was to identify more distinctive and reliable tissue damage or graft function biomarkers, which in the future could increase the pool of transplantable organs by discarding less viable grafts.

Here, we investigate lipid fingerprints at both pre- and post-transplantation, using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) in hepatic tissue in two distinct donor types: *viz.* DBD and DCD, the latter undergoing warm ischaemia events [356] . The main lipid differences between these two donor types were determined by an initial lipidomics screen (112 biopsies), which highlighted 12 targeted phospholipids that were reanalysed in targeted mode and univariate comparisons. After this, associations to clinical outcomes were investigated. The study workflow is illustrated in **Figure 2.1**.

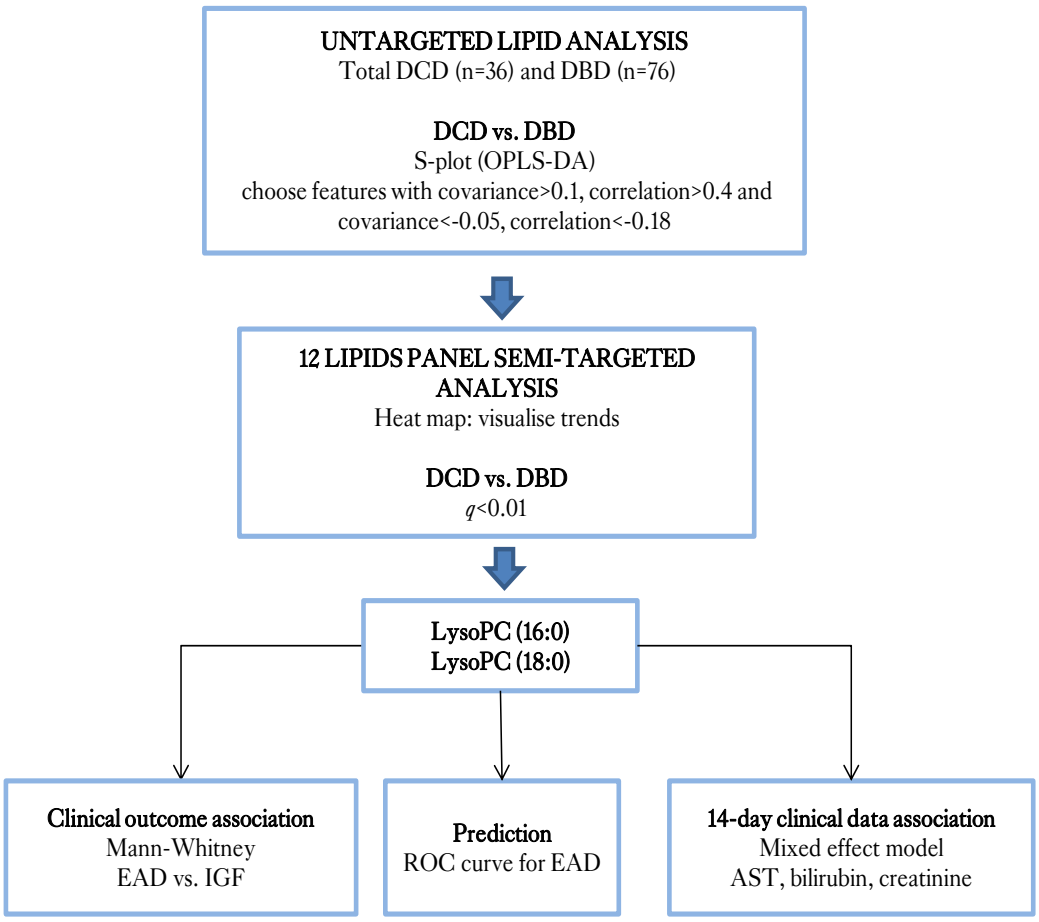


Figure 2.1. Study flowchart illustrates the overall design and analytical procedures from untargeted analysis to targeted analysis and associations of potential biomarkers to clinical outcomes. DCD, donation after circulatory death; DBD, donation after brain death; OPLS-DA, orthogonal projections to latent structures-discriminant analysis; LysoPC, lysophosphatidylcholine; CIT, cold ischaemia time; WIT, warm ischaemia time, EAD, early allograft dysfunction; IGF, Immediate Graft Function; AST, aspartate aminotransferase.

2.2 Methods

2.2.1 Chemicals and reagents

All solvents, water, methanol, ammonium formate and methyl tertiary butyl ether (MTBE), were liquid chromatography – mass spectrometry (LC-MS) grade purchased from Sigma-Aldrich. Two internal standards, heptadecanoic acid (⩾98% purity), tripentadecanoin (⩾98% purity) for the reversed phase, were purchased from Sigma-Aldrich. In-vial dual extractions were performed in amber glass high performance liquid chromatography (HPLC) vials with fixed 0.4 mL inserts (Chromacol: Welwyn Garden City, UK).

2.2.2 Patients and biopsy collection

This study received prior approval from the ethics committee at King's College Hospital, and informed consent was obtained from all subjects. The methods were carried out in accordance with the approved guidelines. Overall 112 Tru-Cut tissue biopsies were obtained from liver allografts pre- and post-transplantation. The first (pre-transplant) biopsy was taken at the end of cold preservation, prior to implantation, and the second (post-transplant) biopsy was obtained approximately 1 hour after graft reperfusion. A separate biopsy was obtained for histopathological evaluation of donor steatosis. Biopsies were immediately snap-frozen in liquid nitrogen and stored at -80˚C until extraction for LC-MS analysis. In all procedures, liver allografts were flash-cooled and perfused with University of Wisconsin preservation fluids until the time of transplantation.

Power calculations were performed for DBD (n=38) and DCD (n=18) participants using 'Gpower3.1' (http://www.gpower.hhu.de/). Assuming a two-sided Type I error of 0.05 and standard normal distributions for lipid molecules, this study has >80% power to detect differences between two groups based on our previous work [353].

2.2.3 Donors

The study included two types of adult donors: DBD (n=38) and DCD (n=18). A wide spectrum of donor clinical data was collected for comparison among groups and for correlation with lipid levels. In the DBD group, 18 of the livers had mild steatosis (up to 30% fat on biopsies), 6 had moderate steatosis (30–60% fat) and the remaining DBD grafts had none. In the DCD group, 2 allografts were mildly steatotic, 6 allografts were moderately steatotic and the remainder were normal. In the DCD group, WIT was calculated from the time when systolic blood pressure was below 50 mmHg to the time of cold perfusion. Total WIT is the sum of Functional WIT, Hepatectomy time and Bench perfusion. The relevant donor data are included in **Table 2.1**.

2.2.4 Recipients

All recipients were patients with stable chronic liver disease who did not require hospitalization prior to transplantation. Indications of liver transplantation in the study include alcoholic liver disease (ALD), primary sclerosing cholangitis (PSC), hepatitis C virus (HCV), hepatocellular carcinoma (HCC), biliary atresia (BA) and others. After

transplantation, all patients received immunosuppressive therapy with tacrolimus and prednisolone. Recipients' 14-day period of international normalized ratio (INR), AST, albumin, gamma-glutamyl transferase (GGT), bilirubin, alkaline phosphatase (ALP) and creatinine level were recorded. Graft performance was assessed based on level of AST, INR and bilirubin levels after transplantation [106]. According to graft performance, recipients were classified into two groups: showing EAD (n=15) and IGF (n=41). The relevant recipient details are listed in **Table 2.1**.

Table 2.1. Summary of clinical data for liver donors and recipients.

Donor		**DBD (N=38)**	**DCD (N=18)**	***p*-value[b]**
Age(years)		53(25-82)	56(35-76)	0.494
Gender(female/male)		22/16	9/9	0.774
Hepatic steatosis	No	14	10	0.470
	Mild (<30%)	18	6	
	Moderate (30-60%)	6	2	
GGT (IU/L) [a]		54(6-208)	62(12-315)	0.686
AST (IU/L) [a]		85(22-517)	137(15-392)	0.281
Bilirubin (µmol/L) [a]		11(3-37)	11(4-26)	0.845
ITU stay (days)		4(1-28)	3(1-10)	NA
Inotrop support (Y/N)		22/16	11/7	1
Functional WIT (min)		NA	21(9-33)	NA
Hepatectomy time (min)			29(13-57)	
Bench perfusion (min)			26(10-44)	
Total WIT (min)			75(46-100)	
CIT (min)		501(210-840)	430(84-720)	0.138
Recipient		**DBD (N=38)**	**DCD (N=18)**	***p*-value[b]**
Age (years)		44(20-65)	55(46-70)	0.003
Gender (female/male)		17/21	6/12	0.563
BMI (kg/m^2)		25.6(18.4-34.6)	26.5(15.9-35.8)	0.357
MELD Score (median)		13	13	NA
ALD		10	8	NA
PSC		6	0	
HCV		1	4	
HCC		1	2	
BA		0	0	
Others		20	4	
AST (IU/L) [a]		1108(114-8075)	1968(309-10054)	0.075
Bilirubin day 7 (µmol/L)		56(6-162)	65(12-175)	0.447
INR day 7		1.04(0.86-1.21)	1.15(0.92-1.96)	0.567
EAD/IGF		8/30	7/11	0.202

DBD, donation after brain death; DCD, donation after circulatory death; GGT, gamma-glutamyl transferase; AST, aspartate aminotransferase; ITU, intensive therapy unit; WIT, warm ischaemia time; CIT, cold ischaemia time; BMI, body mass index; MELD, model for end-stage liver disease; ALD, alcoholic liver disease; PSC, primary sclerosing cholangitis; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; BA, biliary atresia; EAD, early allograft dysfunction; IGF, immediate graft function. Continuous values are expressed as means (minimum-maximum); NA, not applicable. Total WIT is the sum of Functional WIT, Hepatectomy time and Bench perfusion. [a] Tested on the day of operation, [b] Mann Whitney test (2-sided) or Fisher exact test (2-sided).

2.2.5 Lipidomics

Sample preparation for all 112 biopsies and lipidomic analysis followed the procedures below [326]. Between 10 and 30 mg of tissue were obtained and transferred to a pre-weight Eppendorf tube containing a steel bead. Then, 30 µL (per 10 mg of tissue) of a methanol: water mixture (4:1, containing the negative-mode internal standard heptadecanoic acid 10 µg/mL) was added and the samples were homogenized for 5 min at 25 Hz (10 cycles of 0.5 min) in a TissueLyser (Qiagen, MD, US). Following this, 70 µl of the homogenate was added to an amber glass HPLC vial containing a 300 µl glass insert (Chromacol, UK). To this 200 µl of MTBE containing 10 µg/mL of positive-mode internal standard (tripentadecanoin) was added, and the samples were mixed via vortexing at room temperature for 60 min. Then, 30 µl of high purity water was added and samples were centrifuged at 2500g for 20 min at 4 °C. Another 35 µl of the homogenate from each sample was taken to form the pooled QC samples. Same procedures were followed for QCs extraction. 50 µl of the upper lipid-containing MTBE phase from all samples were then transferred to clean vials and injected onto the Waters ACQUITY ultra-performance liquid chromatography-quadrupole time of flight (UPLC-QToF) system directly from the vial. Two different ionization modes including positive and negative were performed for each sample (50 µl for each mode). QC sample was running in between every 8 samples.

Samples were kept in the chamber with targeted temperature of 4 ˚C, and the injection volume is 5 µl with full loop function (20 µl loop size). Chromatographic separation for lipidomics was achieved using an Agilent Poroshell 120 EC-C8 column (150mm × 2.1mm, 2.7 µm), maintained at 55 ˚C. The mass spectrometer (Xevo-QTOF) was

operated in positive and negative ionisation mode. A gradient was employed consisting of 10 mM ammonium formate in water (mobile phase A) and 10mM ammonium formate in methanol (mobile phase B). The solvent was delivered at a flow rate of 0.5 mL/min. For positive mode, the gradient consisted of 0 min (75% B), 23 min (96% B), 36 min (96% B), 36.5 min (100% B), 41.5 min (100% B) and 42 min (75%B). The column was re-equilibrated at 75% B for 9 minutes prior to each injection. For analysis in the negative mode, the gradient started at 75% B increasing linearly to 96% B at 23 min, then increasing further to 99.5% B by 23.1 min and maintained till 30 min, initial conditions were restored at 30.1 min to allow column re-equilibration till 35min. In terms of the mass spectrometric condition, for positive mode, a capillary voltage of 3.2 kV and a cone voltage of 45 V were used. The desolvation gas flow was 400 L/hour and the source temperature was 120˚C. All analyses were acquired using the lock spray to ensure accuracy and reproducibility; leucine enkephalin was used as lock mass (m/z 556.2771 and 278.1141) at a concentration of 200 ng/mL and a flow rate of 10 µL/min. Data were collected in the centroid mode over the mass range m/z 100–1000 with an acquisition time of 0.1 seconds a scan. For negative mode, a capillary voltage of -2.6 kV and a cone voltage of 45 V were used. Desolvation gas flow and desolvation temperature were fixed at 800 L/h and 350 ºC, respectively. The reference solution (leucine enkephalin, m/z 554.2639) was infused with the same conditions describe in the positive mode.

All data was processed within 'XCMS' package in 'R' (version 3.2) and exported into SIMCA version 13 (MKS Umetrics AB, Sweden) for multivariate analysis. Multivariate analysis included pre- and post-transplant matched samples n=112 (DBD n=76, DCD n=36). Donor age, steatosis status, WIT and CIT were included as X-variables, also

described as dependant variables, features or measurements, for multivariate analysis. OPLS-DA was then performed to select features based on covariance p[1] and correlation p(corr) value (p[1]>0.1, p(corr)>0.4 and p[1]<-0.05, p(corr)<-0.18).

2.2.6 Intact Lipid Analysis

Selected lipids were measured in the LC-MS data using Waters MassLynx software (Waters Corporation, Milford, MA), and peak areas were normalized to total ion count ratios. The identification (level 1 confidence) was performed by comparing the structure and fragmentation patterns in the MS^2 data with standards [244, 321, 357].

Median values were used to plot the heat-map using an open source 'R' (version 3.2) with 'gplots' package [358], bean-plot of in each group was drawn in 'beanplot' package[359]. Levels in each group at pre- and post-transplantation stage as well as between DCD and DBD groups were examined with univariate non-parametric Mann-Whitney test (2-sided), and Benjamini- Hochberg correction was applied to control the false discovery rate (FDR) [328]. All p values were represented as q values after correction. Lipids with q<0.01 were selected for clinical correlation analysis.

2.2.7 Clinical correlations

The selected lipids level in both EAD and IGF groups were investigated with Mann-Whitney test (2-sided) to examine the lipids distribution differences, calculations were conducted in SPSS 22 (IBM: Armonk, United States). A prediction model regarding

EAD was built using ROC curve for two combined LysoPCs (LysoPC (16:0) and LysoPC (18:0)) and three combined clinical data (donor AST, donor age and steatosis status) in 'R' with 'pROC' package. The prediction ability of these two curves was assessed by area under curve, accuracy, sensitivity and specificity.

Linear mixed effects models were used to investigate the longitudinal associations between recipients' 14-day AST, bilirubin and creatinine concentrations and each lipid pre-transplant levels. Data were scaled to obtain standard deviation of 1. The average baseline AST, bilirubin and creatinine concentrations and the average change in their concentrations over 14 days (follow-up time) was calculated for all subjects per visit (day) as a group (fixed effects) and subject-specific intercept and slope terms, which reflected deviation from the group average (mixed linear effects) were calculated. An interaction term between visit (day) and lipid pre-transplant levels was used to investigate whether the recipients' longitudinal AST, bilirubin and creatinine concentrations (slope) was associated with lipid pre-transplant levels. All calculations included adjustment for age and gender for the 56 donors. All obtained p values were corrected for multiple comparisons and results given as q values. Linear mixed effect models were performed in Stata/SE13 (StataCorp: Texas, United States).

2.3 Results

2.3.1 Clinical outcomes

There were no significant differences between DBD and DCD groups in donors' ages, EAD/ immediate graft function (IGF) distribution, liver enzymes, hepatic steatosis or serum bilirubin levels. Differences were observed in the recipients' ages (p<0.01) between these two groups (**Table 2.1**).

2.3.2 Lipidomics multivariate analysis and selected phospholipids

To discover relevant lipid features, a PCA model (R^2X=0.592, Q^2=0.521) was initially constructed, although no obvious difference was observed. Then, an orthogonal projection to latent structures-discriminant analysis (OPLS-DA) model (**Figure 2.2**) among DBD and DCD grafts was built with n=112. The model's figures of merit were R^2X=0.659, R^2Y=0.941 and Q^2=0.58. R^2X explains a feature percentage (65.9%) which is explained by this model, R^2Y indicates that 94.1% of the group variance is interpreted, and Q^2 shows the prediction ability of the model with 58%. 7-fold cross validation (CV) (CV p value=$6.20x10^{-12}$) suggested its reliability. From the model, 12 features differentiated among 2 groups with p[1]>0.1, p(corr)>0.4 and p[1]<-0.05, p(corr)<-0.18 were selected (**Table 2.2**). These selected 12 features consisted of 2 lysophosphatidylethanolamines (LysoPEs), 2 lysophosphatidylcholines (LysoPCs), 6 phosphatidylcholines (PCs) and 2 phosphatidylethanolamines (PEs). Donor age, steatosis status, functional warm ischaemia time (WIT) and cold ischaemia time (CIT)

showed low p[1] and p(corr) values and were not chosen as important variables. A heat map was computed to visualise trends for the 12 selected lipids (**Figure 2.3**).

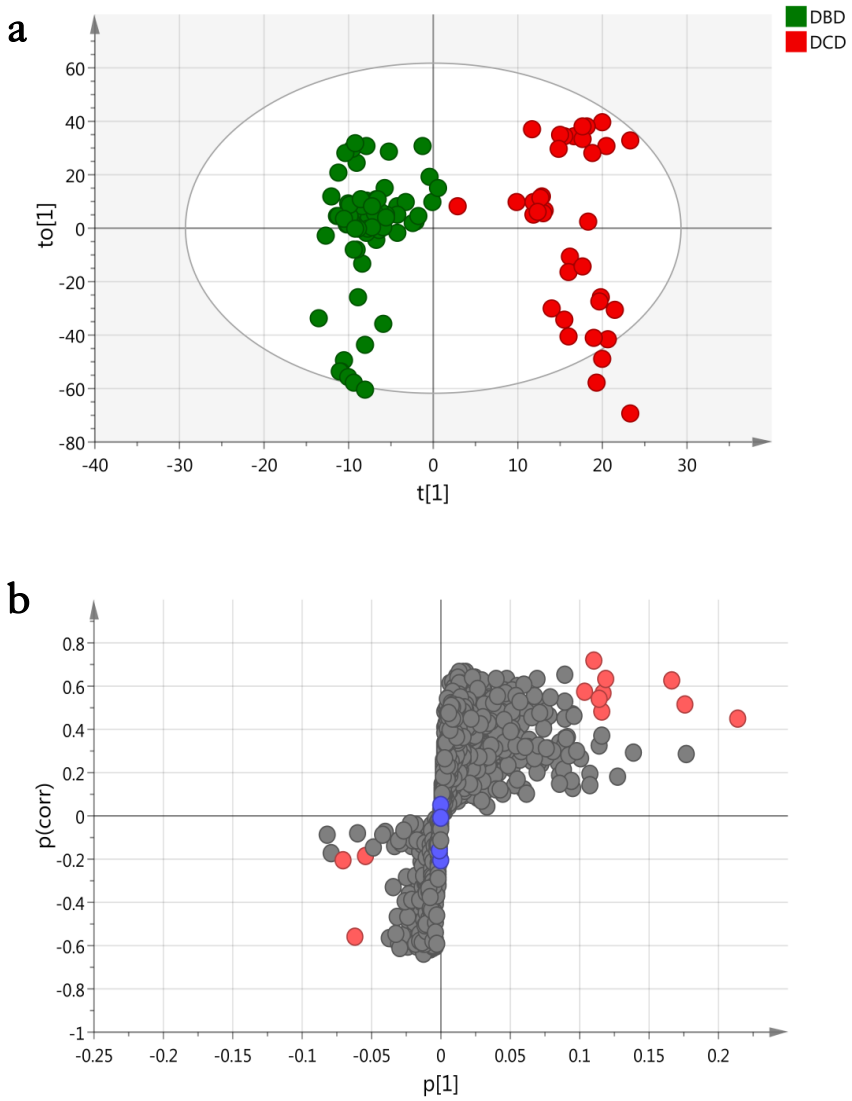


Figure 2.2. OPLS-DA model from screen of lipidomics with n=112 (DBD=76, DCD=36) biopsies. a) Score plot of the model, R^2X=0.659, R^2Y=0.941, Q^2=0.58, cross validation p value=6.20x10^{-12}; b) S-plot of the model, features with p[1]>0.1, p(corr)>0.4 and p[1]<-0.05, p(corr)<-0.18 were selected (red circles), variables including donor age, steatosis status, WIT and CIT were clustered in the middle (blue circles). DCD, donation after circulatory death; DBD, donation after brain death; WIT, warm ischaemia time; CIT, cold ischaemia time.

Table 2.2. Identification of markers based on molecular weight, retention time and collision induced dissociation fragmentation of 12 lipids.

Identified as	Retention time (min)	m/z	Observed ion	QC RSD (%)
LysoPE (16:0)	3.46	452.28	$[M-H]^-$	9.91
LysoPE (18:0)	5.24	480.31	$[M-H]^-$	7.86
LysoPC (16:0)	3.42	496.33	$[M+H]^+$	10.9
LysoPC (18:0)	5.16	524.37	$[M+H]^+$	20.3
PE (34:2)	16.81	714.51	$[M-H]^-$	4.61
PE (38:4)	19.32	750.55	$[M-H]^-$	4.51
PC (34:2)	16.73	758.56	$[M+H]^+$	19.5
PC (36:4)	16.80	782.57	$[M+H]^+$	8.92
PC (36:3)	17.38	784.59	$[M+H]^+$	7.66
PC (36:2)	18.41	786.61	$[M+H]^+$	10.6
PC (38:4)	18.46	810.60	$[M+H]^+$	1.77
PC (38:3)	19.01	812.62	$[M+H]^+$	11.4

LysoPE, lysophosphatidylethanolamine; LysoPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

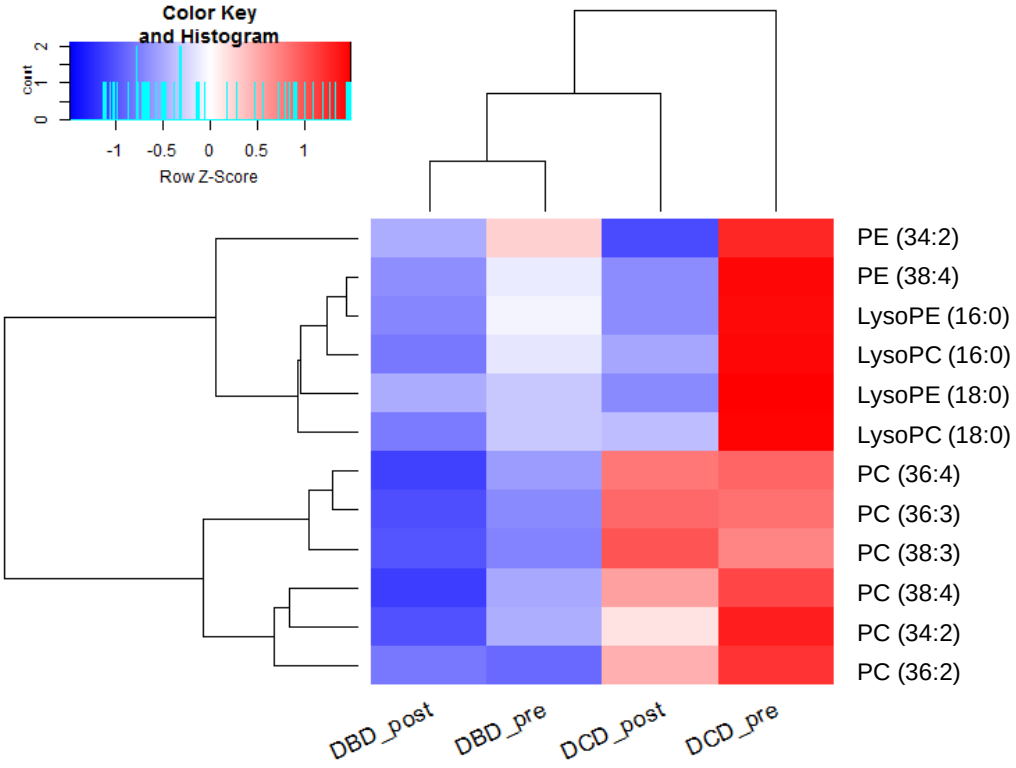


Figure 2.3. Heat-map showing distinct lipid profiles of DBD and DCD tissue in 112transplant samples. Values are median amounts per donor group at pre- and post-transplantation stages. A clustering analysis (dendrogram) shows which lipids differ most; red depicts high amounts and blue means low level. DCD, donation after circulatory death; DBD, donation after brain death; PE, phosphatidylethanolamine; PC, phosphatidylcholine; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine.

2.3.3 Targeted analysis of phospholipids per donor type during transplantation

From the heat map, no obvious differences were observed in the DBD group from pre- to post- for all 12 lipids except for PE (34:2). All lipids were more abundant in DCD at pre-transplantation stage compared with DBD. All 6 PCs levels remained constant from pre to post in the DCD group while 2 PEs, 2 LysoPEs and 2 LysoPCs showed lower concentration at post- transplant stage.

12 lipids were reanalysed from the semi-quantified data for univariate analysis. The result of Mann-Whitney test with multiple comparison correction revealed that 2

lysophosphatidylcholines showed significant differences at pre- transplantation stage (q=0.002 and q=0.003 respectively) between the two donor groups. **Figure 2.4** shows the amounts for LysoPC (16:0) and LysoPC (18:0), **Figure 2.4a** and **b** respectively show elevated levels in DCD when comparing to DBD for both LysoPCs (q<0.01).

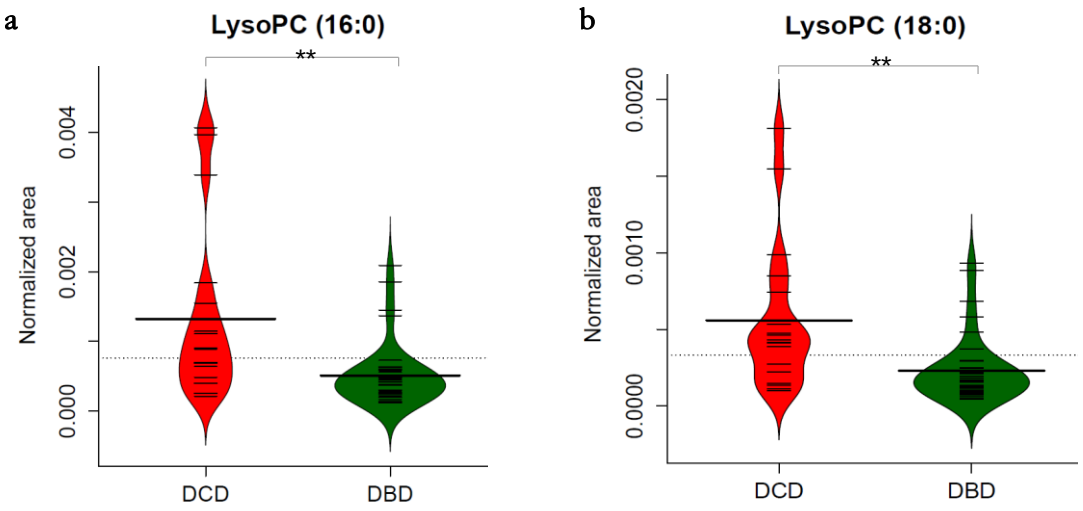
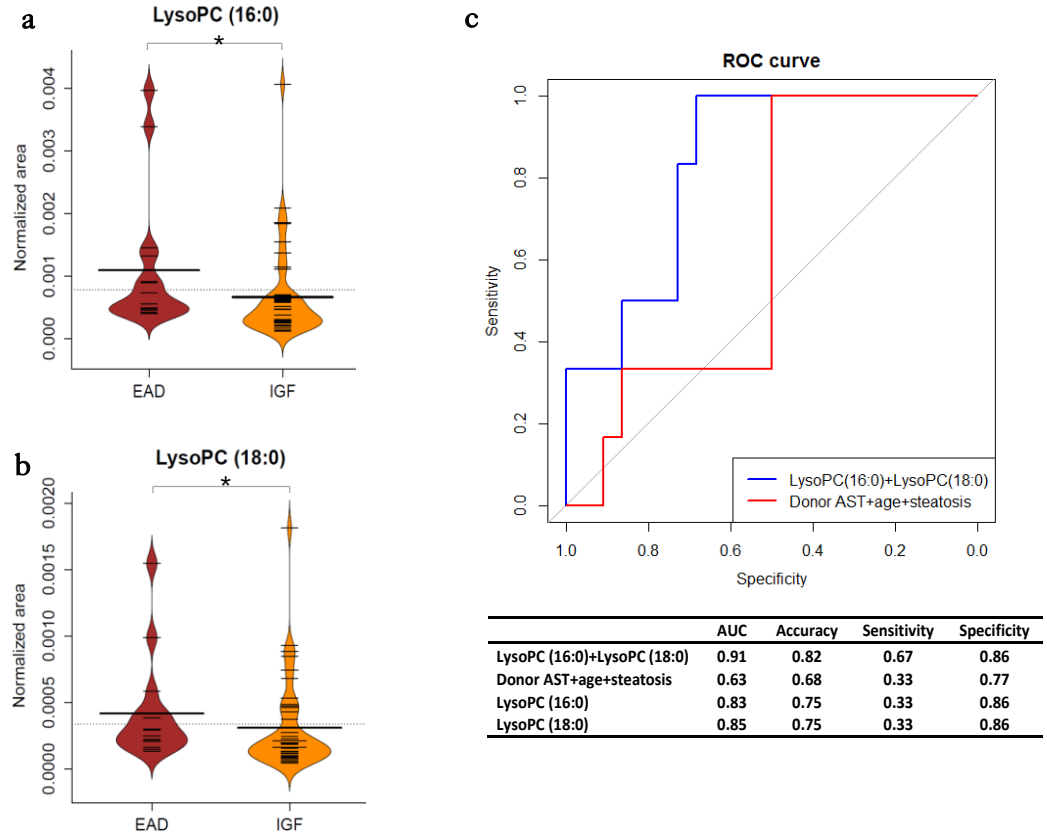


Figure 2.4. Bean-plots showing variation in 2 lysophosphocholines among DBD and DCD at the pre-transplantation stage. a) LysoPC (16:0); b) LysoPC (18:0). Mann-Whitney 2-sided, ** is q<0.01, q value is p value adjusted by Benjamini- Hochberg FDR correction DCD, donation after circulatory death; DBD, donation after brain death; LysoPC, lysophosphatidylcholine.

2.3.4 Correlation of selected lysophospholipids to clinical data

The distribution of 2 lysophosphatidylcholines in EAD (n=15) and IGF (n=41) groups was investigated. The Mann-Whitney test illustrated that the amount of LysoPC (16:0) and LysoPC (18:0) at pre-transplant were significantly higher in the EAD group (p=0.013 and p=0.03 respectively) (**Figure 2.5a and b**). The prediction ability of LysoPCs (LysoPC (16:0) and LysoPC (18:0)) and clinical parameters (donor AST, donor age and steatosis status) was evaluated by receiver-operating characteristics

(ROC) curve. The area under the curve (AUC) for LysoPCs group was 0.91 (accuracy=0.82, sensitivity=0.67, specificity=0.86). For comparison, the AUC for three pre-transplant clinical parameters was 0.63 (accuracy=0.68, sensitivity=0.33, specificity=0.77) (**Figure 2.5c**).



	AUC	Accuracy	Sensitivity	Specificity
LysoPC (16:0)+LysoPC (18:0)	0.91	0.82	0.67	0.86
Donor AST+age+steatosis	0.63	0.68	0.33	0.77
LysoPC (16:0)	0.83	0.75	0.33	0.86
LysoPC (18:0)	0.85	0.75	0.33	0.86

Figure 2.5. Two LysoPCs amounts, a) LysoPC (16:0) and b) LysoPC (18:0), showing significant differences between EAD (n=15) and IGF (n=41) groups; c) ROC curve prediction of EAD based on two LysoPCs and three donor clinical parameters. Mann-Whitney 2-sided, * is p<0.05; LysoPC, lysophosphatidylcholine; EAD, early allograft dysfunction; IGF, immediate graft function.

The effect of the two-selected pre-transplant lysophosphatidylcholines levels on biomedical parameters including AST, bilirubin, and creatinine levels within 14-day post-transplant was examined by mixed-effects maximum likelihood regression followed by Benjamini- Hochberg correction. Overall 6 models were built with the

combination of one biomedical parameter and one lipid in each model adjusted for age and gender. Fitting was deemed adequate for all 6 models ($p<0.0001$ from chi-square test). After the multiple comparison correction, significant associations were observed between longitudinal changes in AST concentration (slope) and LysoPC (16:0) levels (LysoPC (16:0)×day interaction ($q<0.05$)), and between longitudinal changes in AST concentration (slope) and LysoPC (18:0) levels (LysoPC (18:0)×day interaction ($q<0.05$)). For instance, with every standard deviation (SD) increase in LysoPC (16:0)×day interaction, recipients' AST levels, on average, decreased 0.017 IU/L (with 95% confidence interval subscripts: LysoPC (16:0)×day interaction: $_{-0.032}$-0.017$_{-0.003}$ p=0.006). The model parameters are listed in **Table 2.3** and the effects of LysoPCs concentrations on AST levels are present in **Figure 2.6.**

Table 2.3 Summary of mixed-effects models between AST concentration and LysoPCs level, day, interaction of LysoPC level and day.

AST		Coefficient	95% confidence interval	*p*-value
Model 1	LysoPC (16:0)	0.122	-0.068, 0.313	0.21
	day	-0.0003	-0.016, 0.015	0.97
	LysoPC (16:0) × day	-0.017	-0.032, -0.003	**0.006**
Model 2	LysoPC (18:0)	0.117	-0.073, -0.308	0.229
	day	-0.0003	-0.016, 0.015	0.965
	LysoPC (18:0) × day	-0.02	-0.035, -0.005	**0.007**

LysoPC, Lysophosphatidylcholine; AST, aspartate aminotransferase.

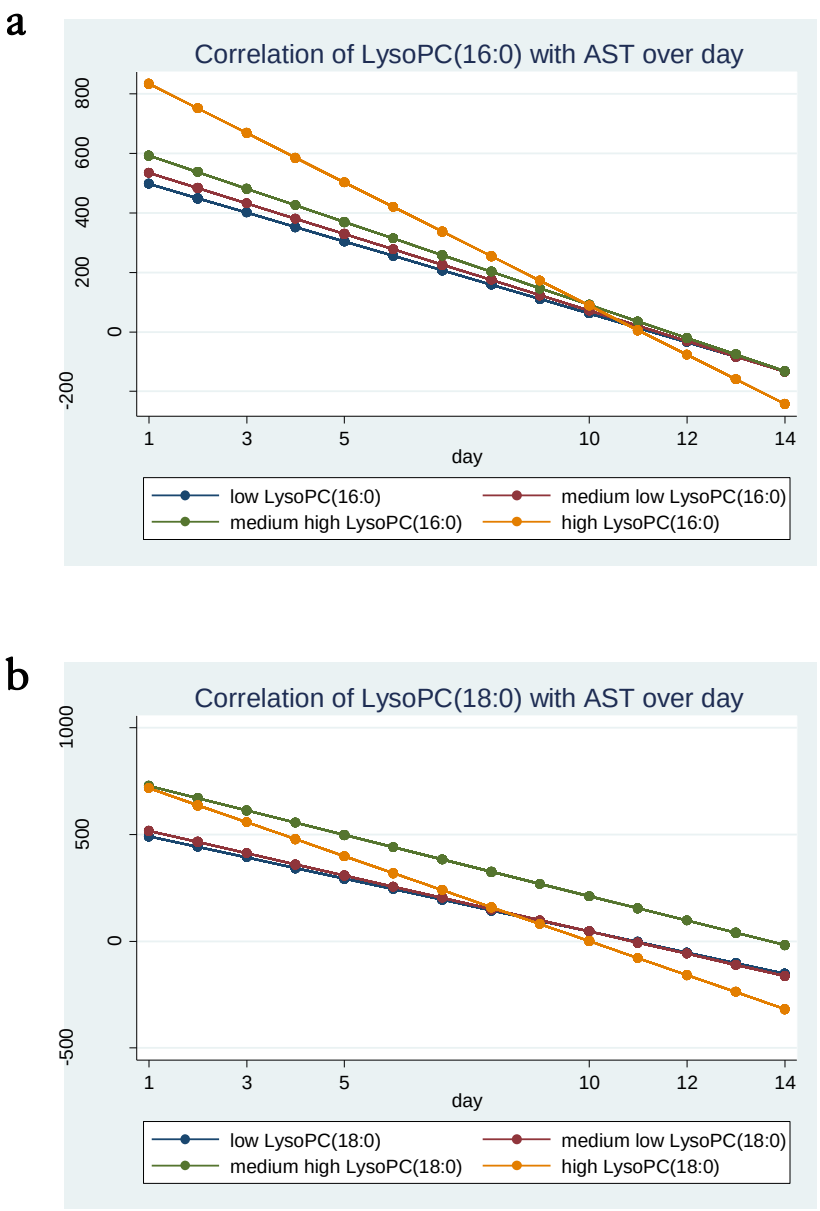


Figure 2.6. Correlation of two pre-transplant lysophospholipids level and post-transplant AST, bilirubin and creatinine during 14-day period in mixed-effects model. a) Correlation of LysoPC (16:0) and AST over 14-day; b) Correlation of LysoPC (18:0) and AST over 14-day. LysoPC, Lysophosphatidylcholine; AST, aspartate aminotransferase.

2.4 Discussion

The urgent need for specific molecular markers of hepatic tissue quality has given rise to small molecule phenotyping studies investigating a range of liver pathologies [2, 55]. The study reported here is the first study to distinguish lipid profiles between two different liver-donor types, with correlations to clinical outcomes related with liver graft dysfunction and clinical follow-up. Matched liver tissue biopsies were obtained at both pre- and post-transplantation stages from two types of donors, *viz.* DBD and DCD. DBD livers may suffer inflammatory changes in relation to brain death and ITU management and undergo a significant period of cold preservation following retrieval. DCD donors have no diagnosis of brain death; however, livers from DCD undergo an additional period of warm ischaemia prior to retrieval. This ischaemia period has been previously associated with increased rates of graft failure and associated with both short and long-term complications following transplantation [347]. A substantial number of DCD organs are thus discarded because of the lack of precise assays to evaluate transplant outcomes.

Inflammatory responses in donor liver biopsies are donor-type specific, DBD tissue showed elevated levels of pro-inflammatory changes at the pre-transplantation stage. This was attributed to inflammatory events associated with brain death in the donors [54]. Following reperfusion, DBD tissue showed high levels of neutrophil infiltration and deposition of activated platelets. On the other hand, DCD allografts demonstrated lower inflammatory response but higher cell death rates that correlated with the length of warm ischaemia [54, 360]. Since increasing cell death was observed in DCD, we hypothesised that lipid cell death mediators could be affected during transplant[53].

By focusing on donor-type using lipidomics for discovery, we identified 12 lipids differentiating among DBD and DCD. Trends were illustrated in the heat-map (**Figure 2.3**) for the lipid median values in pre- and post- transplantation across the two donor types. This panel of lipids did not change from pre- to post-transplant in the same donor type, implying that lipid changes observed at pre-transplantation are likely to be related to ischaemia damage rather than reperfusion effects.

PCs were more abundant in DCD at both transplant stages. Phosphatidylcholines have been associated with inflammation [361]. However, contradictory data shows that these lipids have a protective function as studies have linked them to regeneration processes in the liver [362-364].

12 lipids were measured again, and univariate analysis was applied. Lipids with statistical significance when comparing DBD and DCD in both pre- and post-transplant biopsies were LysoPC (16:0) (q<0.01) and LysoPC (18:0) (q<0.01) showing higher values in DCD (**Figure 2.4**).

LysoPCs are lysophospholipids generated from PCs (**Figure 2.7**) via the action of phospholipase A_2, and like other lysophospholipids they are likely to be activators or inhibitors of G-protein coupled receptors (GPCRs) [365]. Phospholipase A_2 synthesises lysophospholipids including both lysoPCs and lysoPEs from PEs and PCs by producing free fatty acids.

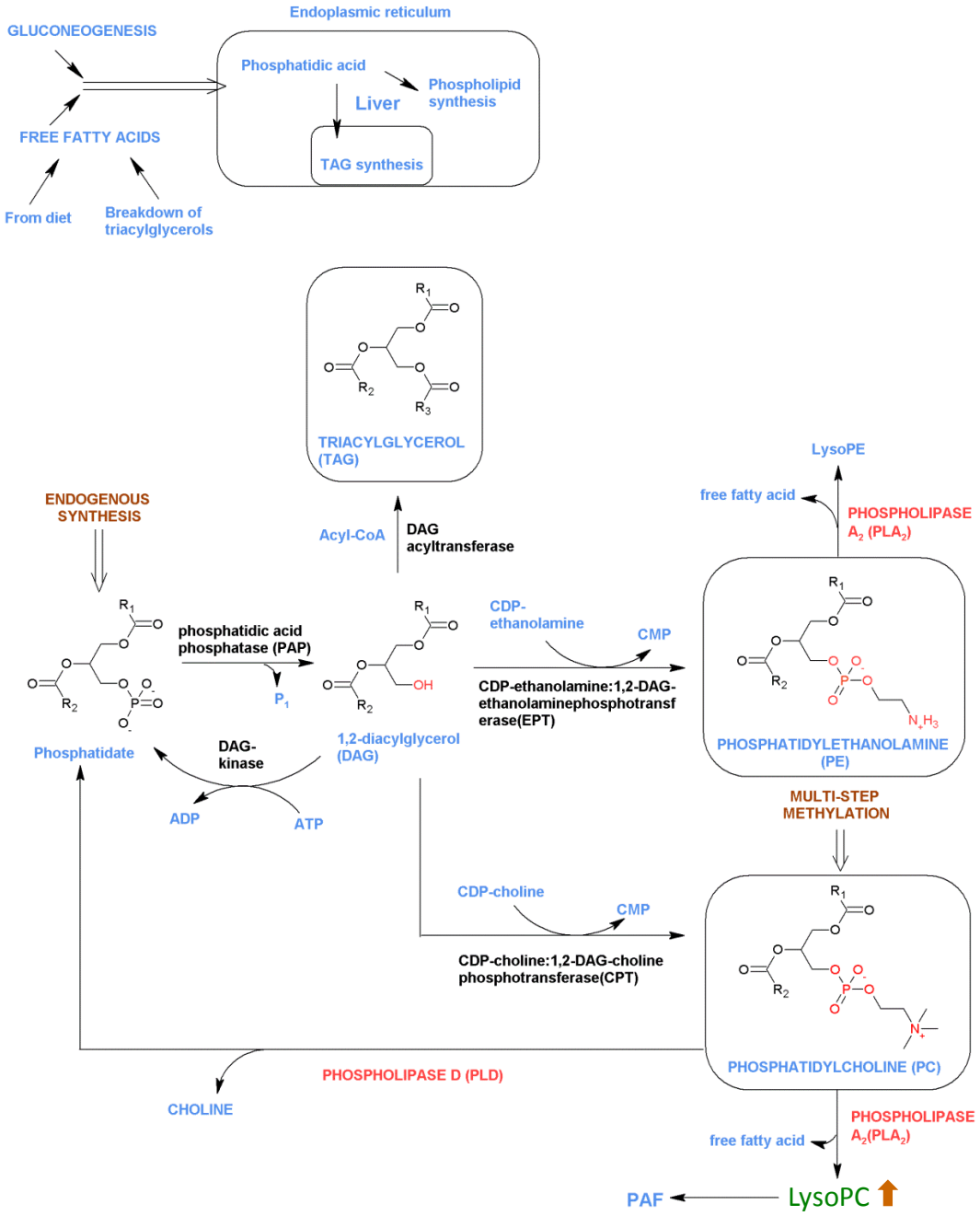


Figure 2.7. Lipid metabolism in the healthy liver, showing endogenous metabolism of triacylglycerol (TAG) and phospholipids. Phosphatidic acid (PA) is generated in vivo by catabolic modification of glycerol-3-phosphate. Diacylglycerol (DAG) is a key metabolic intermediate and intracellular signalling molecule which can be converted to TAGs by the action of DAG acyltransferases to glycerophospholipids including phosphatidylcholines (PC), phosphatidylethanolamines (PE) and ultimately to lysophosphatidylcholines (LysoPC), lysophosphatidylethanolamines (LysoPE) by the action of phospholipase A_2. LysoPCs showed higher amounts in donation after circulatory death (DCD) group in this study.

In DCD donors, LysoPCs were found to be increased. LysoPCs are known to be precursors of the platelet-activating factor (PAF), a potent phospholipid inflammatory, which has been previously associated with both hepatic ischaemia and reperfusion injury (IRI) [366-368]. Hence, DCD donors might be prone to the PAF mediated inflammatory pathway before transplantation (pathway illustrated in **Figure 2.7**).

The *in-vivo* function of LysoPCs and in particular their role in intracellular signalling is mostly unknown. LysoPCs have previously been associated with inflammatory liver disease [369, 370]. Interestingly Cortes *et al.* [55] found in a study where all donors were from DBD that LysoPC (16:0) and LysoPC (18:0) were higher in grafts that presented EAD $p<0.05$, supporting the hypothesis that these lysophospholipids affect transplant outcomes.

To understand the relevance of the two lipids to transplantation we investigated EAD. EAD is a clinical term which can reflect donor, recipient and transplant characteristics, and it can be utilized as a transplant benchmark [371]. The incidence of EAD in our study was 26%, this was in the upper range of known values for incidence ranging from 14% to 27% [372]. LysoPC (16:0) and LysoPC (18:0) showed ($p<0.05$) significant difference when their contents between EAD and IGF groups were compared. The ROC curve of these two LysoPCs versus three clinical parameters known to be risk factors of EAD indicated that LysoPC (16:0) and LysoPC (18 :0) were better at prediction of EAD, in particular, accuracy increased from 68% for AST, age and steatosis to 82% for LysoPCs. A limitation of this comparison is that it was applied to compare donors only at pre-transplantation; however it is known that EAD is affected by multiple factors including the recipients and the surgery procedures [373].

The regression between AST 14-day concentrations and two LysoPCs indicated that LysoPCs levels at pre-transplant were associated with AST concentration at post-transplant. Since AST is considered an indicator of liver injury, the association between high AST post-transplantation and LysoPCs pre-transplantation warrants further investigation of LysoPC (16:0) and LysoPC (18 :0) as markers of liver damage.

2.5 Conclusion

In conclusion, the analysis of phospholipids in the context of liver transplantation has identified two lipids differentiated in DBD and DCD livers. LysoPC (16:0) and LysoPC (18:0) could have a role as intermediates in signalling tissue damage due to warm ischaemia. This study is relevant in identifying pre-transplant biomarkers for tissue quality and in designing appropriate therapeutic strategies in order to minimize damage related to ischaemia injury.

Chapter 3 Investigation of ceramide mediated inflammation during ischaemia reperfusion injury in liver allografts

The named researchers below contributed to this chapter by conducting the following experiments.

1. Mr Wayel Jassem (King's College Hospital, UK): Biopsies collection and clinical data collection.
2. Mr Parthi Srinivasan (King's College Hospital, UK): Biopsies collection.
3. Mr Nigel Heaton (King's College Hospital, UK): Biopsies collection.

3.1 Introduction

In recent decades, the prevalence of end stage liver diseases has greatly increased due to factors such as alcohol abuse, unhealthy lifestyle and microbiome disturbance [374]. As a result, the increasing demands for suitable organ donors for liver transplantation exceed the number of donors, which has remained largely static [375]. The worsening organ shortage is reflected in the median time to transplant in wait-listed adult patients. In the United States median time increased from 14.8 months in 2004 to 19.5 months in 2011. As such, transplant centres and allocation organizations have attempted to expand the pool of acceptable donors, including the use of DCD donors [376]. Although some centres have reported satisfactory results by using DCD allografts, other data indicates that recipients of controlled DCD liver allografts have an increased incidence of graft dysfunction, early graft loss and cholangiopathy as compared to recipients of DBD livers [377-381].

The pathophysiology of cardiac death is markedly different from that of brain death. As compared to livers obtained from DBD, in which there is no consistent proceeding cardiac arrest, DCD livers are subjected to additional hypoxic insult. However, brain death generates an inflammatory response with the release of various pro-inflammatory mediators, leading to upregulated expression of adhesion molecules on vascular endothelium and subsequent leukocyte tissue infiltration [22, 382, 383]. Jassem *et al.* demonstrated that prior to transplantation DCD allografts have lower expression of ICAM-1, potentially suggesting less allograft inflammation [384].

Liver ischaemia reperfusion injury (IRI) is the injury caused by the ischaemia and reperfusion process in liver retrieval, transporting and implanting. The damage is

sustained during the cold preservation after liver retrieval and during warm reperfusion at implantation in recipients. IRI is associated with the release of reactive oxygen species and pro-inflammatory mediators [385]. Studies revealed that sphingolipids metabolites regulate a diverse range of cellular processes that are important in inflammatory disorders and hepatic insulin resistance [85, 386]. Among several types of sphingolipids, the ceramide pathway is of interest as it is associated with inflammatory response and it is also widely distributed in mammalian tissue [387]. Elevated levels of ceramides promote inflammation and downstream apoptosis by enhancing susceptibility to palmitate-induced cell death [85, 388, 389]. The role of ceramides has yet to be described in human liver transplantation [85] despite previous observations of ischaemia reperfusion-induced accumulation of ceramides in various organs, including the liver [388, 389].

In this study, ceramide contents were assessed to examine the IRI in biopsies obtained from DBD and DCD before and after transplantation.

3.2 Methods

3.2.1 Patient sampling

Overall 46 Tru-Cut tissue biopsies were obtained from liver allografts pre- and post-transplantation. The first (pre-transplant) biopsy was taken at the end of cold preservation, prior to implantation, and the second (post-transplant) biopsy was obtained approximately 1 hour after graft reperfusion. A separate biopsy was obtained for histopathological evaluation of donor steatosis. Biopsies were immediately snap-frozen in liquid nitrogen and stored at -80˚C until extraction for LC-MS analysis. In all procedures, liver allografts were flash-cooled and perfused with University of Wisconsin preservation fluids until the time of transplantation. This study received prior approval from the ethics committee at King's College Hospital.

The study included 2 types of adult donors: DBD (n=10) and DCD (n=13). A wide spectrum of donor clinical data was collected for comparison among groups and for correlation with lipid level. In the DBD group, 2 of the livers had mild steatosis (up to 30% fat on biopsies), 3 had moderate steatosis (30–60% fat) and the remaining DBD grafts had none. In the DCD group, 4 allografts were mildly steatotic, and the remainder were normal. In the DCD group, functional warm ischaemia time (WIT) was calculated from the time when systolic blood pressure was below 50 mmHg to the time of organ retrieval. The relevant donor data are included in **Table 3.1**.

All recipients were patients with stable chronic liver disease who did not require hospitalization prior to transplantation. Indications of liver transplantation in the study include alcoholic liver disease (ALD), primary sclerosing cholangitis (PSC), hepatic C virus (HCV), hepatocellular carcinoma (HCC), biliary atresia (BA) and others. After

transplantation, all patients received immunosuppressive therapy with tacrolimus and prednisolone. Recipients' 14-day period of international normalized ratio (INR), aspartate aminotransferase (AST), albumin, gamma-glutamyl transferase (GGT), bilirubin, alkaline phosphatase (ALP) and creatinine level were recorded. Graft performance was assessed based on level of AST, INR and bilirubin level after transplantation. The relevant recipient details are listed in **Table 3.2**.

3.2.2 Sample preparation and analysis

For all 46 biopsies, identical lipidomics procedures for sample preparation and negative LC-MS/MS analysis were executed. Sample preparation prior to analysis followed description in **Chapter 2.2.5**.

3.2.3 Ceramides analysis

The identification of ceramides Cer16, Cer18, Cer20, Cer22 and Cer24 (with the number suffix denoting the length of the acyl chain) was achieved by structure and fragmentation patterns comparison of the MS^2 data in databases, literature and C8 standard [244, 321, 357, 390]. Five ceramides were measured in the LC-MS data using Waters MassLynx software (Waters Corporation, Milford, MA) and their peak area ratios to internal standard were calculated. Mean ratio values were used to plot the heat-map using an open source 'R' package, 'gplots' [358]. Subsequently univariate non-parametric Mann Whitney test was performed to examine the mean difference of each

ceramide level in each group at pre- and post-transplantation stage, as well as between DBD and DCD at both stages.

Spearman's correlation analysis was applied separately in DCD and DBD groups to investigate the correlation of ceramides at both pre- and post-transplant with recipients' 14-day clinical data including INR, GGT, AST, ALP, ALB, Bilirubin, creatinine and donors' age, gender, ITU stay, inotrope support, WIT, CIT. All obtained *p* values were adjusted for multiple comparisons using Benjamini- Hochberg correction to control the false discovery rate (FDR) [328]. All statistical calculations were conducted in SPSS 22 (IBM: Armonk, United States). Orthogonal projections to latent structures-discriminant analysis (OPLS-DA, SIMCA 13.0.2, Umetrics, Sweden) was used for multivariate analysis using the 5 ceramides as variables. Two models were investigated, DBD vs. DCD (n=46), and DBD vs. DCD for liver biopsies from non-steatosis (n=28). Details of the analytical workflow are provided in the **Figure 3.1**.

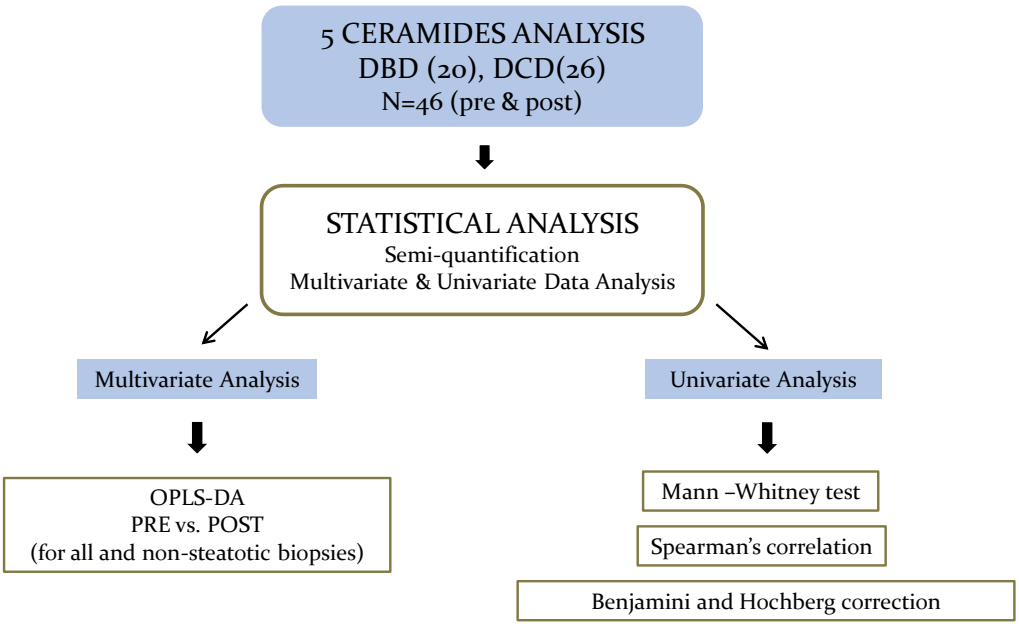


Figure 3.1. Analytical workflow for five ceramides. DBD, donation after brain death; DCD, donation after circulatory death; OPLS-DA, orthogonal projections to latent structures-discriminant analysis.

3.3 Results and discussion

3.3.1 Clinical outcomes

No significant differences were observed in all parameters listed in **Table 3.1** and **Table 3.2** when comparing DBD with DCD. DCD livers underwent an additional total WIT of 72±18 min (average ± SD) in 13 donors, although 21% shorter average CIT were found in DCD.

Table 3.1. Summary of clinical data for liver donors.

Donor		DBD (N=10)	DCD (N=13)	*p*-value[b] DBD vs DCD
Age(years)		51(19)	54(13)	0.69
Gender(female/male)		7/3	7/6	0.67
Hepatic steatosis	No	5	9	0.13
	Mild (<30%)	2	4	
	Moderate (30-60%)	3	0	
GGT (IU/L) [a]		35(14)	28(19)	0.73
AST (IU/L) [a]		39(6)	70(53)	1
Bilirubin (µmol/L) [a]		14(13)	8(4)	0.79
ITU stay (days)		5(9)	2(2)	NA
Inotrop support (Y/N)		7/3	9/4	NA
Functional WIT (min)			20(7)	NA
Hepatectomy time (min)			27(10)	
Bench perfusion (min)			26(12)	
Total WIT (min)			72(18)	
CIT (min)		496(212)	389(117)	0.60

DBD, donation after brain death; DCD, donation after circulatory death; GGT, gamma-glutamyl transferase; AST, aspartate aminotransferase; ITU, intensive treatment unit; WIT, warm ischaemia time; CIT, cold ischaemia time; NA, not applicable. Continuous values are expressed as means (standard deviation). Total WIT is the sum of Functional WIT, Hepatectomy time and Bench perfusion. [a] Tested on the day of operation, [b] Mann Whitney test (2-sided) or Fisher exact test (2-sided).

Table 3.2. Summary of clinical data for liver recipients

Recipient	DBD (N=10)	DCD (N=13)	*p*-value[b] DBD vs DCD
Age (years)	42(13)	57(6)	0.56
Gender (female/male)	7/3	4/9	0.10
BMI (kg/m^2)	24(4)	26(5)	0.34
MELD Score (median)	12	13	1.0
Alcoholic liver disease (ALD)	2	9	
Primary sclerosing cholangitis (PSC)	2	0	
Hepatic C virus (HCV)	1	5	NA
Hepatocellular carcinoma (HCC)	0	2	
Biliary atresia (BA)	0	0	
Others	5	1	
AST (IU/L) [a]	963(611)	2356(2714)	0.17
Bilirubin day 5 (μmol/L)	80(74)	74(70)	0.92
INR day 2	1.43(0.16)	1.51(0.46)	0.65

DBD, donation after brain death; DCD, donation after circulatory death; BMI, body mass index; MELD, model for end-stage liver disease; ALD, alcoholic liver disease; PSC, primary sclerosing cholangitis; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; BA, biliary atresia; AST, aspartate aminotransferase; INR, international normalized ratio; NA, not applicable. [a] Tested on the day of operation, [b] Mann Whitney test (2-sided) or Fisher exact test (2-sided).

3.3.2 Ceramide identification

There are two production pathways for ceramides (**Figure 3.2**). De novo ceramide synthesis is achieved with the participation of ceramide synthases (CerS). Apart from this, ceramides are also produced by sphingolipids recycling pathway [388]. Based on the exploration of ceramide synthases (CerS) distribution by northern blot, some groups found that the main three ceramide synthases that exist in mouse liver are CerS2 (75%),

CerS4 (20%) and CerS5 (5%) [388, 391]. It is also found that short chain ceramides with fatty acyl chains contain fewer than 12 carbons are served as detergents as they can easily disperse in water. Ceramides with long fatty acyl chains of 16-28 carbons are more common in mammalian cellular membranes [392]. Of these ceramide syntheses, CerS2 and CerS4 mainly synthesize C20:0, C22:0, C24:1, C24:0, C26:1 and C26:0 ceramides. CerS5 is in charge of C14:0, C16:0, C18:0 and C18:1 ceramides [388].

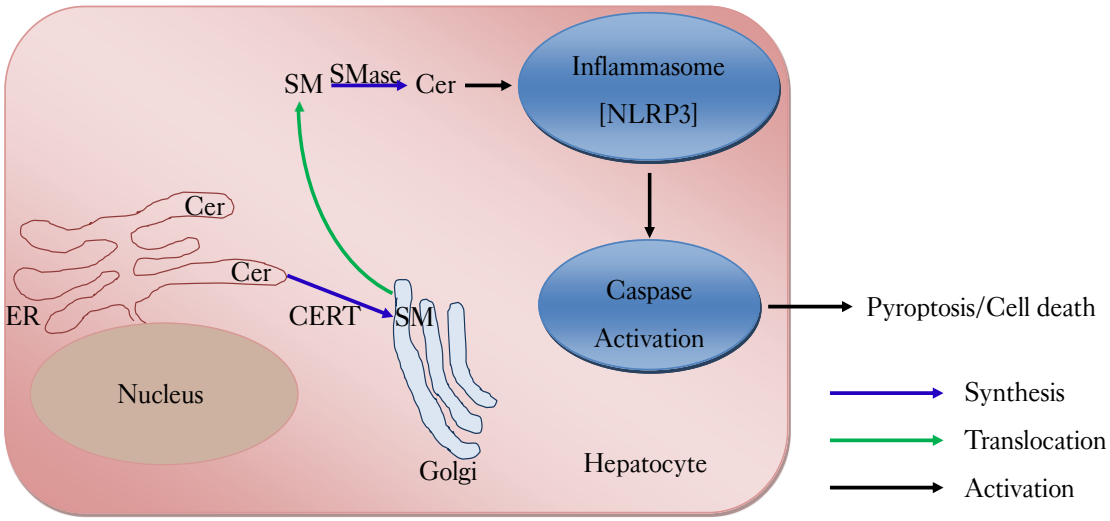


Figure 3.2. Ceramide synthesis pathways in hepatocyte. ER, endoplasmic reticulum; Cer, ceramide; CERT, ceramide transfer protein; SM, sphingomyelin; SMase, sphingomyelinase. Figure conceptualised from the linkage of sphingolipids and inflammatory disease[85].

Ceramides were identified based on suggested fragmentation patterns and comparison with a standard Ceramide (d18:1/8:0) [390]. The speculative structures of two daughter ions (m/z 168.18 and 237.28) are listed in **Figure 3.3**. The chromatogram and spectra (both channel 1 and channel 2) of the Cer8 were shown below in **Figure 3.4**. The two main parent ions of Cer8 are $[M+FA-H]^-$ and $[M-H]^-$ with the m/z of 470.51 and 424.49, respectively.

After the study of possible fragments of the standard, an example of Cer24 fragmentation is illustrated in **Figure 3.5**. The expected two fragments were observed as in highlighted red circle. **Table 3.3** contains the relevant analytical data for the ceramide analysis (list of mass-to-charge ratio or m/z values, the observed ions, main fragments and the instrumental variation of the measurements in quality control samples).

As shown in the spectra of Cer8, the most abundant ion was in the format of $[M-H]^-$, while $[M+FA-H]^-$ was the main parent ion in Cer24. The fragmentation pattern difference might be caused by the side chain length in these two ceramides. However, the $[M+FA-H]^-$ was found to be the main parent ion in all five ceramides included in this study.

Table 3.3. Identification of markers based on molecular weight, retention time and collision induced dissociation fragmentation of lipids.

m/z	Identified as	Observed ion	Fragments	RSD (%) QC
582.51	Cer (d18:1/16:0)/Cer16	$[M+FA-H]^-$	237, 280	22.9
610.54	Cer (d18:1/18:0)/Cer18	$[M+FA-H]^-$	237, 308	14.3
638.57	Cer (d18:1/20:0)/Cer20	$[M+FA-H]^-$	237, 336	19.1
666.60	Cer (d18:1/22:0)/Cer22	$[M+FA-H]^-$	237, 364	4.93
694.64	Cer (d18:1/24:0)/Cer24	$[M+FA-H]^-$	237, 392	3.85

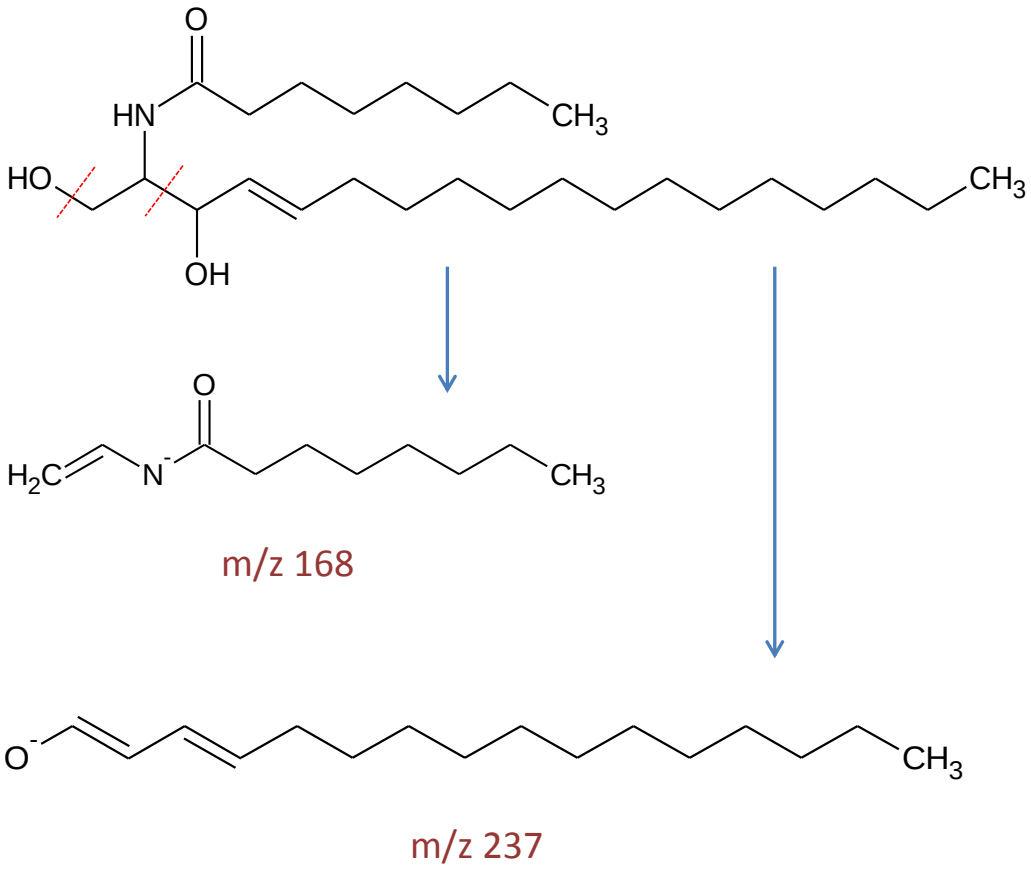


Figure 3.3. Suggested structures of two daughter ions of the Cer8 standard.

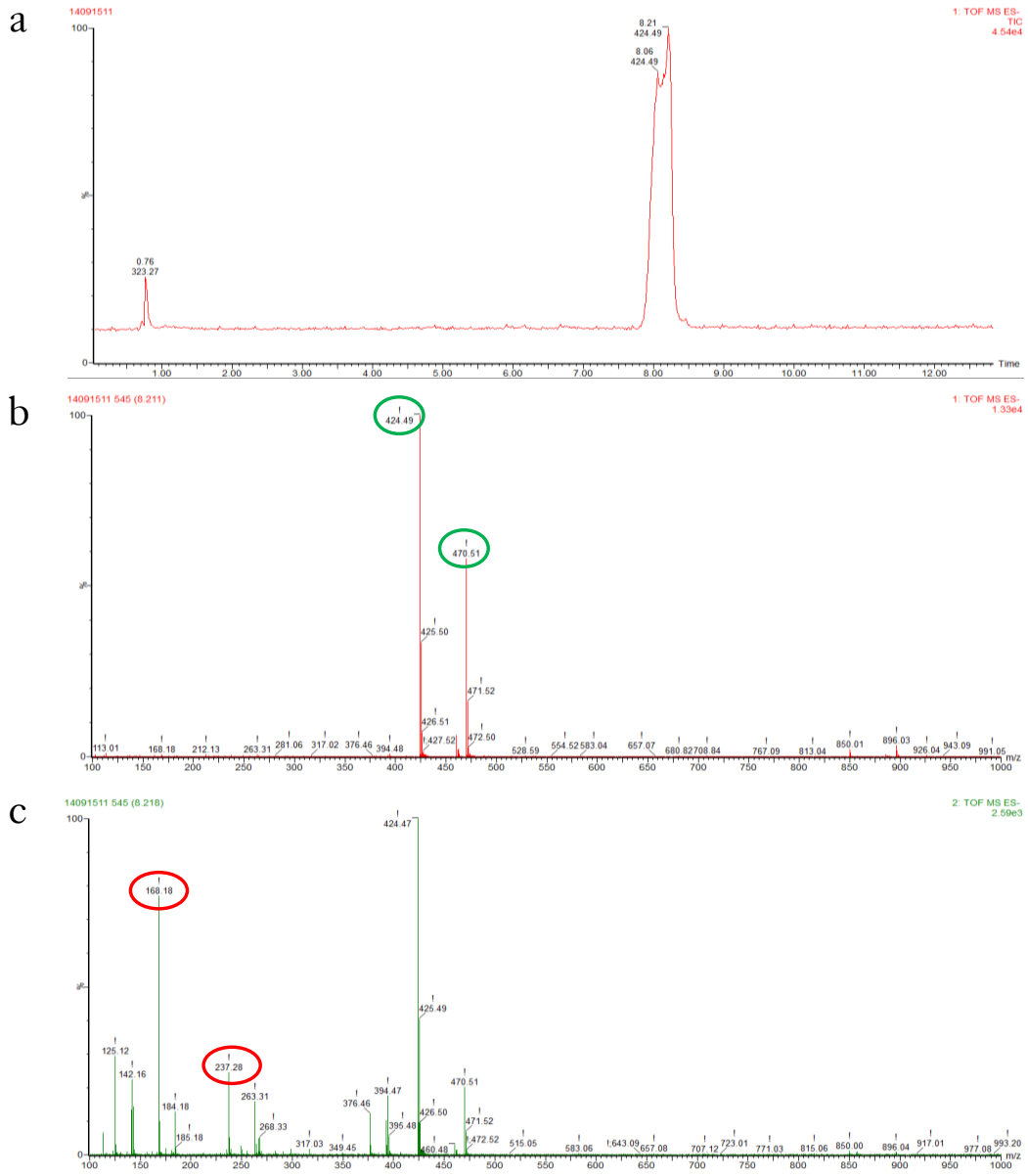


Figure 3.4. The chromatogram and spectra of the Cer8 standard. (a) Chromatogram of C8 ceramide standard, (b) Channel 1 MS spectrum of C8 ceramide showing two main parent ions, (c) Channel 2 MS spectrum of C8 ceramide with daughter ions.

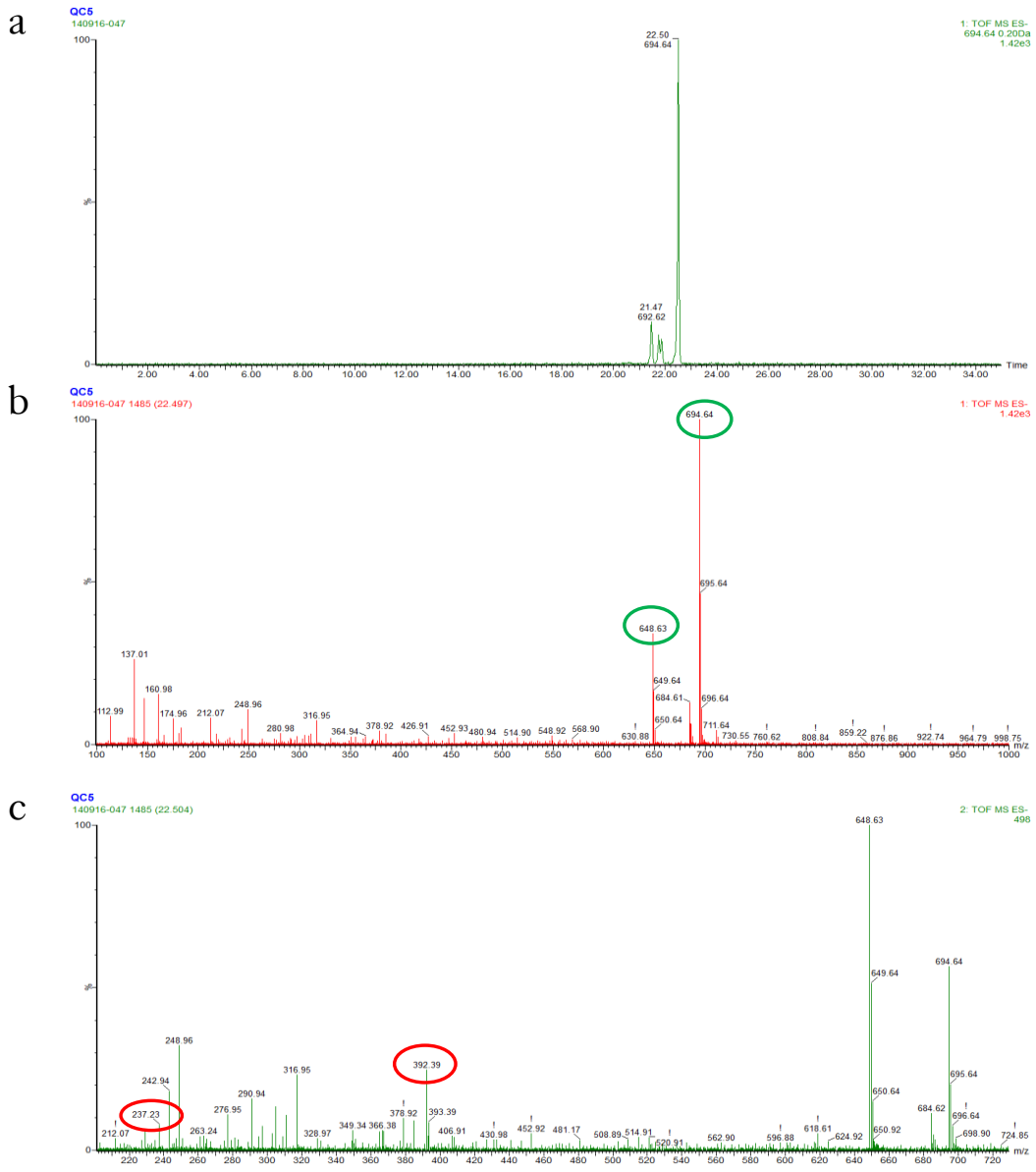


Figure 3.5. The chromatogram and spectra of the Cer24 in a QC sample. (a) Chromatogram of C24 ceramide, (b) Channel 1 MS spectrum of C24 ceramide showing two main parent ions, (c) Channel 2 MS spectrum of C24 ceramide with daughter ions.

3.3.3 Univariate analysis for ceramides per donor type during transplant

The amounts for the 5 selected ceramides were measured and plotted in a heat-map dendrogram (**Figure 3.6**). When comparing DBD-pre to DCD-pre, no significant

differences were observed for all five ceramides, and the same result applies to DBD-post and DCD-post.

To assess the impact of ischaemia injury, ceramide levels in all 46 biopsies were compared between pre- and post-transplantation within each donor group. The analysis showed that significant difference was observed in regard to ceramides level from pre- to post-transplantation. Overall changes in DBD from pre- to post transplantation for C18, C20, C24 ($p<0.05$) were significant and C22 ($p<0.05$) were more pronounced for DCD suggesting that the inflammation response was more severe in DBD.

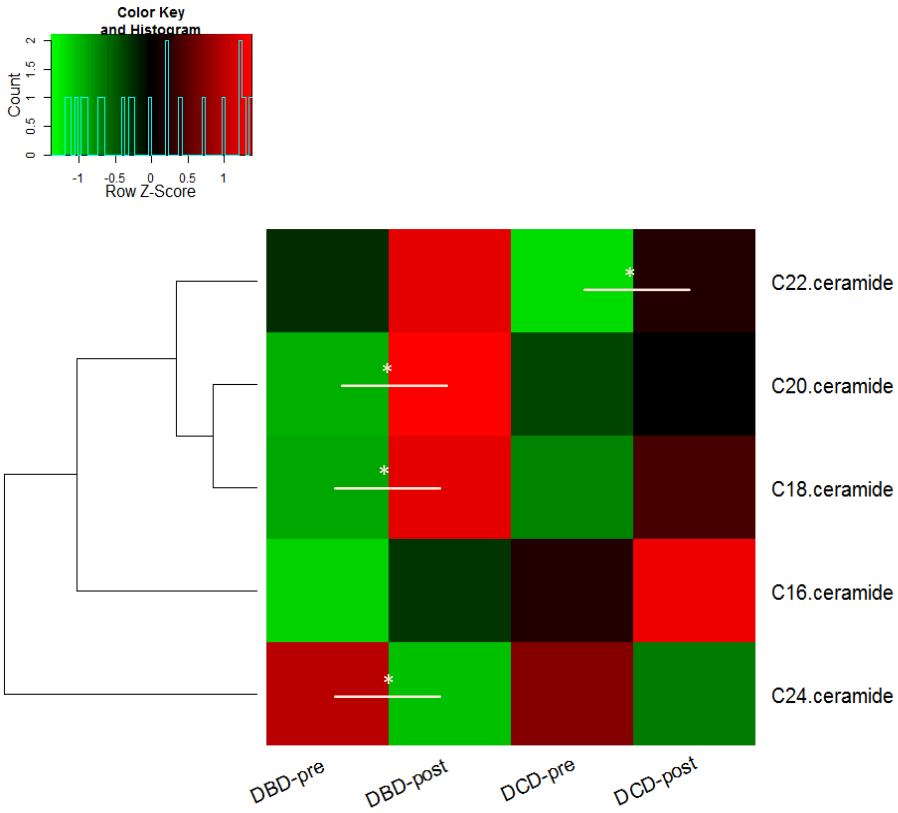


Figure 3.6. Heat-map showing distinct ceramides profiles of DBD and DCD tissue in 46 transplant samples. Values are mean amounts per donor group at pre and post-transplantation stages. A clustering analysis (dendrogram) shows which lipids differ most; red depicts increased amounts and green decreased.

Ceramides are at present well known markers of inflammation and elevated ceramide level are thought to produce inflammation and downstream apoptosis by enhancing susceptibility to palmitate-induced cell death [85]. In I/R injury, accumulation of ceramides was observed in organ tissues, however it is not yet known how the size of a ceramide determines inflammation response [388, 389]. In-vitro experiments showed that elevated level of C16-ceramide resulted in TNF-α-induced hepatocyte apoptosis [393]. Conversely elevated de-novo C18-ceramide, synthesised by CerS4 in the liver, was also found to function as a powerful pro-apoptotic activator in tumour cells [386, 391]. Bigger ceramides also called very long chain ceramides (C24-ceramides) are thought to be the most abundant in healthy liver and their decrease can in turn indicate liver pathology [394]. As illustrated in **Figure 3.2**, After being generated by enhancement of de novo biosynthesis or sphingolipid recycling, ceramide can then signal to trigger the Nlrp3 inflammasome. The stimulation of which promotes the activation of caspase 1, leading to pyroptosis, a highly inflammatory form of programmed cell death [395].

These results are in line with what was expected from IR response, increased long chain (C18-C20) and decreased very long chain (C24) suggesting higher inflammation at post-transplant stage. C22 increased its level after graft implantation; this is not in agreement with an inflammation response considering it is also a very long chain ceramide.

3.3.4 Correlation of ceramides to clinical data

Spearman's rank correlation analysis was applied separately in DCD and DBD groups to investigate the correlation of ceramides at both pre and post-transplant with recipients' 14-day clinical data. According to the correlation result, creatinine, INR, bilirubin, donor age and WIT show high significance, then Benjamini- Hochberg false discovery correction was used to test the p value of those 5 parameters. After the p-value correction, Cer18 showed significant correlation to bilirubin and INR at pre-transplantation, creatinine at post-transplantation in DCD, while no significant correlation reflects in DBD group at both pre and post-transplantation (see details in **Table 3.4**).

Table 3.4. Correlation of ceramides (n=23) to clinical outcomes.

DCD-pre	C18-ceramide		DCD-post	C18-ceramide	
	Corr. coefficient	q value		Corr. coefficient	q value
BIL 3	0.7747**	0.0031	INR 2	0.7785**	0.0017
BIL 9	0.8182**	0.0011	INR 4	0.7565**	0.0028
BIL 10	0.8252**	0.0010	INR 8	0.8421***	0.0006
BIL 11	0.9423***	0.00005	CREAT 9	0.8476***	0.0005
BIL 12	0.9000***	0.0009	CREAT 10	0.8476***	0.0005
BIL 13	0.9643***	0.0005			

Spearman correlations and Benjamini-Hochberg correction was applied. **q<0.01, ***q<0.001. DCD, donation after circulatory death; DBD, donation after brain death; BIL, bilirubin; INR, international ratio; CREAT, creatinine.

Lang *et al.* found that bilirubin induces ceramide formation [396], which explains the high correlation of bilirubin and Cer18 at DCD pre-transplant stage in our study. Moreover, as bilirubin is an antioxidant that can stimulate apoptosis of various cells [397,

398], the higher Cer18 level in DCD at pre-transplant stage compared with DBD indicates that severe apoptosis took place in DCD hepatocytes. INR, the assessment parameter for coagulation monitoring [399], indicated the liver injury when its value is elevated, hence, the correlation of INR with Cer18 in DCD post-transplant demonstrates ischaemia reperfusion injury in DCD.

The association between C18-ceramide and serum level of creatinine is of interest as renal dysfunction post-transplant has been seen to occur at higher rate in liver transplantation from DCD [400].

3.4 Conclusion

This study explored five widely distributed ceramide levels (Cer16, Cer18, Cer20, Cer22 and Cer24) in 46 biopsies from two different donor groups, DBD and DCD, at both pre- and post-transplantation stages. It was discovered that ceramide levels differ in DBD and DCD, as well at two transplantation stages suggesting that the ceramide-mediated inflammation response was more severe in DBD. Correlation between ceramides with clinical outcomes revealed that ischaemia reperfusion injury in DCD is related with enzymes and clinical index. In conclusion, ischaemia reperfusion injury involved during liver transplantation in DBD and DCD impacts the levels and trends of ceramides, revealing that diverse extent of inflammation were regulated in two groups

Chapter 4 Purines associated with liver tissue quality before transplantation

The named researchers below contributed to this chapter by conducting the following experiments.

1. Mr Wayel Jassem (King's College Hospital, UK): Biopsies collection and clinical data collection.
2. Mr Parthi Srinivasan (King's College Hospital, UK): Biopsies collection.
3. Mr Nigel Heaton (King's College Hospital, UK): Biopsies collection.

4.1 Introduction

There is an ever increasing demand of organs for transplantation, but the available organs are insufficient to meet the demand [401]. This is reflected by the static number of people registered on the Organ Donor Register (ODR) in the UK which only saw a 5% rise from 2012 to 2017 [402]. Meanwhile in the same period, the number of patients requiring a liver transplant increased from 452 to 519 [402]. This stark surge in the demand for liver transplants is attributable to the global incidence of liver diseases such as drug-induced hepatotoxicity [403], fatty liver diseases [404], cirrhosis [405] and hepatitis infections, primarily caused by hepatitis B and C virus [406, 407].

To safeguard patients, pre-transplant donor screening is used to determine the plausible success rate of liver transplants. The optimal/sub-optimal criteria for liver donors including age (<50 years/>50 years), weight (<100kg/>100kg), intensive care stay (<5 days/>5 days), functional warm ischaemic time (<20 mins/>20mins, <30mins), cold ischaemia time (<8 hours/>8 hours, <12 hours) and steatosis (<10%/>15%) [19]. This has resulted in up to 20% of donation after brain death (DBD) organs not meeting the strict clinical criteria [408] and a 78% increase in the discard rate of donation after circulatory (DCD) livers [409]. This has potentially led to a number of otherwise transplantable organs being discarded [410]. Therefore, the need to identify more specific pre-transplantation markers of liver damage is a priority in order to expand the pool of transplantable livers.

Currently, liver function tests are the standard assessment in establishing liver dysfunction, as evaluated by elevated concentrations of liver-enzymes such as alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST)

and gamma-glutamyl transferase (GGT) [411, 412]. However, such tests lack sensitivity and specificity and can be affected by patient factors such as genetics, medicines and other non-associated diseases [413-416]. Thus far, transcriptomics and genomics have been used to discover biomarkers in liver pathophysiology [417]. It has been postulated in experimental studies that an association exists between brain death and an increased inflammatory response in DBD livers leading to early allograft dysfunction (EAD). There is no clear evidence of brain death itself entailing a systemic inflammatory response, but the adenine-monophosphate (AMP) pathway has been highlighted in inflammation [418]. Furthermore, the association of the AMP pathway to immediate graft function (IGF) has not been studied previously [419, 420].

In **Chapter 2**, two Lysophosphatidylcholines, were found to be differentiated in DBD and DCD groups at pre- transplantation stages, as well as increased in EAD. The objective of this study was to employ hydrophilic interaction chromatography-mass spectrometry (HILIC-MS) technique to investigate polar metabolites at both pre- and post-transplantation, in hepatic tissue in two distinct donor types: *viz.* DBD and DCD; the latter undergoing warm ischaemia events. Following this, I wanted to ask whether metabolites that were different between donor types were associated to clinical outcomes *viz.* EAD and IGF. The study workflow is illustrated in **Figure 4.1**.

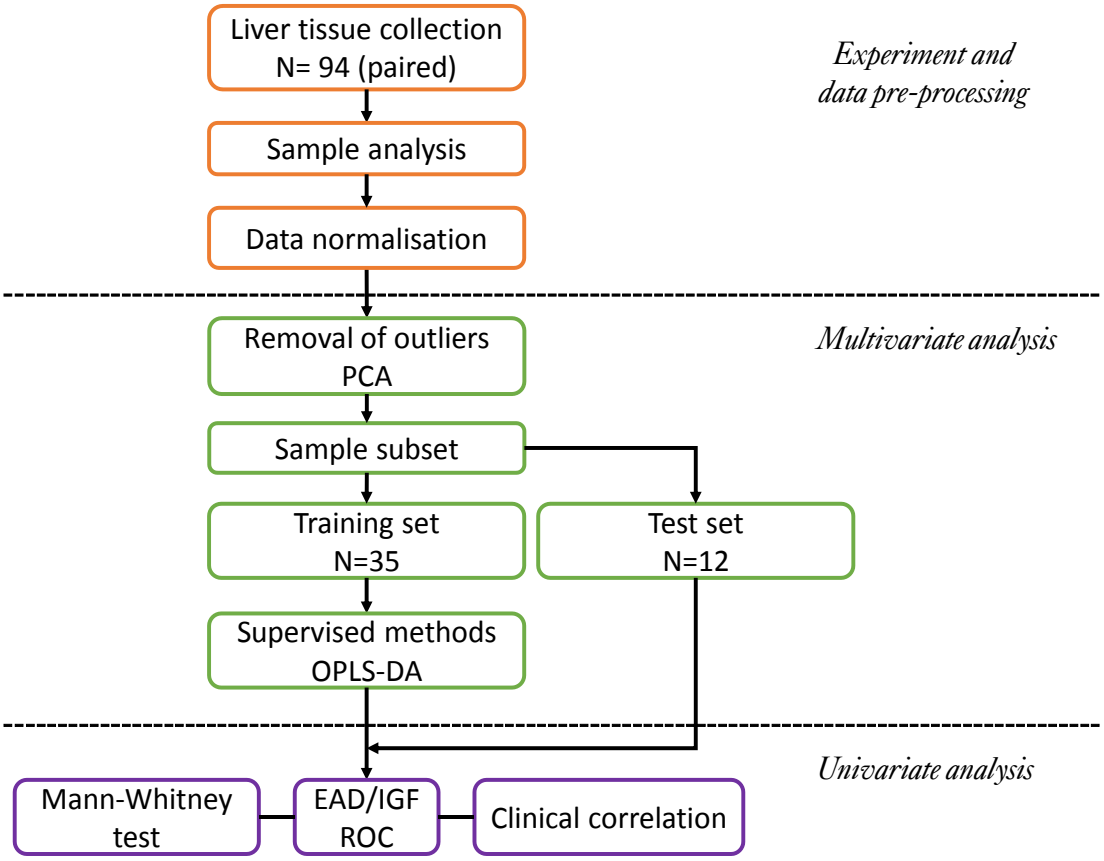


Figure 4.1. Study workflow. PCA, principal component analysis; OPLS-DA, orthogonal projections to latent structures-discriminant analysis; EAD; early allograft dysfunction; IGF, immediate graft function; ROC, receiver operating characteristic.

4.2 Methods

4.2.1 Chemicals and reagents

All solvents, water, methanol, acetonitrile, formic acid and methyl tertiary butyl ether (MTBE), were liquid chromatography–mass spectrometry (LC-MS) grade purchased from Sigma-Aldrich. Two internal standards, L-serine$^{13}C_3{}^{15}N$ (95%) and L-valine$^{13}C_3{}^{15}N$ (95%) for hydrophilic liquid interaction chromatography (HILIC) were purchased from Sigma-Aldrich. In-vial dual extractions were performed in amber glass high performance liquid chromatography (HPLC) vials with fixed 0.4 mL inserts (Chromacol: Welwyn Garden City, UK).

4.2.2 Patients and samples

All patients and samples information were stated in **Chapter** 2.2.2, 2.2.3 and 2.2.4. Following the transfer of organic phase from extracted sample for lipidomic analysis (**Chapter** 2.2.5), 100 µl the lower aqueous phase from all samples were also transferred to clean vials for further sample preparation. To this, 10ul methanol: water (4:1) solution containing 1mmol/ml internal standards (L-serine$^{13}C_3{}^{15}N$ and L-valine$^{13}C_3{}^{15}N$) were added. After being vortexing for 10 mins at room temperature, samples were centrifuged at 3000g for 30 min at 4 °C. Same procedures were applied to quality controls which were the aliquots from mixture of all samples.

4.2.3 Chromatographic and spectrometric conditions

Samples were kept in the chamber with targeted temperature of 4 ˚C, and the injection volume is 5 µl with full loop function (20 µl loop size). Chromatographic separation for HILIC analysis was achieved using a Merck Sequant ZIC-HILIC column (150mm × 4.6mm, 5 µm) coupled to a Merck Sequant ZIC-HILIC guard column (20mm × 2.1mm), maintained at room temperature. A gradient was employed consisting of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The solvent was delivered at a flow rate of 0.3 mL/min. The gradient started at 80% mobile phase B, followed by a linear reduction to 20% mobile phase B after 30 minutes, initial conditions were restored at 30.1min to allow 9.9 minutes of column re-equilibration. The mass spectrometer (Xevo-QTOF) was operated in positive ion mode, and a capillary voltage of 3.2 kV and a cone voltage of 35 V were used. The desolvation gas flow was 500 L/hour and the source temperature was 120˚C. All analyses were acquired using the lock spray to ensure accuracy and reproducibility; leucine enkephalin was used as lock mass (m/z 556.2771 and 278.1141) at a concentration of 200 ng/mL and a flow rate of 10 µL/min. Data were collected in the centroid mode over the mass range m/z 50–1000 with an acquisition time of 0.1 seconds a scan. QCs were run in between every 8 samples in random orders.

4.2.4 Multivariate analysis

All data was processed within 'XCMS' package in 'R Studio' (version 1.0.153), the multivariate analyses were conducted in both 'R Studio' and 'SIMCA' (version 14, MKS Umetrics AB, Sweden) for. Multivariate analysis included pre- and post-

transplant matched samples n=94 (DBD n=70, DCD n=24) and 17 QCs. Principal component analysis (PCA) was carried out to detect outlier(s) and to examine the distribution of QCs.

All pre- transplant data was then divided into training dataset (DBD n=30, DCD n=5) and test dataset (DBD n=5, DCD n=7). Orthogonal projections to latent structures-discriminant analysis (OPLS-DA) model was built based on the training dataset to examine the profiling of pre- transplant samples in DBD and DCD groups. Test dataset was utilised to assess the prediction ability of the built model. S-plot derived from the OPLS-DA model was then applied to select features based on covariance p[1] and correlation p(corr) value (p[1]>0.1, p(corr)>0.4 and p[1]<-0.1, p(corr)<-0.4).

4.2.5 Univariate analysis

Selected features were measured in the LC-MS data using Waters MassLynx software (Waters Corporation, Milford, MA). Feature concentrations were expressed as ratios of lipid peak areas to internal standard peak area. The identification (level 2 confidence) was performed by using metabolites mass to search against in-house and public metabolite databases [244, 321, 357], their structure and fragmentation patterns in the MS^2 data were also studied by comparing with databases to aid the annotation.

Feature levels between DBD and DCD as well as between EAD and IGF groups at pre- and post-transplantation stage were examined with univariate non-parametric Mann-Whitney test (2-sided) and Benjamini-Hochberg test to control the false discovery rate (FDR). To follow this, correlation analyses between annotated metabolites and clinical

features (AST, Bilirubin, GGT) were conducted. Calculations were conducted in SPSS 23 (IBM: Armonk, United States). Figures were plotted in GraphPad Prism 6 (GraphPad, US).

4.3 Results

4.3.1 Clinical outcomes

Patients details on all 94 samples including comparisons on the clinical variables between the groups are represented in **Table 4.1**. There were no significant differences between DBD and DCD groups in donors' ages, EAD/ IGF distribution, liver enzymes, hepatic steatosis or serum bilirubin levels. Differences were observed in the recipients' ages (p<0.05) between these two groups.

Table 4.1. Demographic characteristics and clinical data for 94 subjects involved in this study.

Donor		**DBD (N=35)**	**DCD (N=12)**	***p*-value[b]**
Age(years)		53(18-82)	56(35-76)	0.526
Gender(female/male)		19/16	6/6	1
Hepatic steatosis	No	14	7	0.305
	Mild (<30%)	18	3	
	Moderate (30-60%)	3	2	
GGT (IU/L) [a]		52(6-208)	92(21-315)	0.342
AST (IU/L) [a]		85(22-517)	161(15-392)	0.139
Bilirubin (μmol/L) [a]		11(3-37)	12(4-26)	0.695
ITU stay (days)		4(1-28)	4(1-10)	0.168
Inotrop support (Y/N)		19/16	6/6	1
Functional WIT (min)			21(9-33)	
Hepatectomy time (min)			31(13-57)	
Bench perfusion (min)		NA	28(10-44)	NA
Total WIT (min)			79(46-100)	
CIT (min)		504(210-840)	457(270-720)	0.212
Recipient		**DBD (N=35)**	**DCD (N=12)**	***p*-value[b]**
Age (years)		44(20-65)	54(46-70)	0.029
Gender (female/male)		13/22	5/7	1
BMI (kg/m^2)		25.8(18.4-34.6)	27.3(22.1-35.8)	0.277
MELD Score (median)		12.5	11.5	NA
ALD		9	3	NA
PSC		5	0	
HCV		1	2	
HCC		1	2	

BA Others	0 19	0 5	
AST (IU/L) [a]	480(10-7485)	613(18-5307)	0.494
Bilirubin day 7 (μmol/L)	56(7-258)	52(12-103)	0.772
INR day 7	1.04(0.85-1.21)	1.06(0.92-1.3)	0.909
EAD/IGF	6/29	4/8	0.251

DBD, donation after brain death; DCD, donation after circulatory death; GGT, gamma-glutamyl transferase; AST, aspartate aminotransferase; ITU, intensive therapy unit; WIT, warm ischaemia time; CIT, cold ischaemia time; BMI, body mass index; MELD, model for end-stage liver disease; ALD, alcoholic liver disease; PSC, primary sclerosing cholangitis; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; BA, biliary atresia; EAD, early allograft dysfunction; IGF, immediate graft function. Continuous values are expressed as means (minimum-maximum); NA, not applicable. Total WIT is the sum of Functional WIT, Hepatectomy time and Bench perfusion. [a] Tested on the day of operation, [b] Mann Whitney test (2-sided) or Fisher exact test (2-sided).

4.3.2 Multivariate model and feature selection

The unit variance (UV) scaled dataset was first inspected for detection of outlier(s). In **Figure 4.2**, PCA score plot and observation diagnostic plot (observations exceed both score distance and orthogonal distance are strong outliers, as located in the top right area of the plot) showed one strong outlier, sample 2_DCD_post_24, and was removed. After excluding the outlier, no obvious outlier was discovered as shown in **Figure 4.3**. After the removal of one outlier, the distribution of QCs and samples were investigated. As can be seen in **Figure 4.4**, QCs clustered near the origin in the PCA plot (R^2X=0.391, Q^2=0.361).

To follow this, comparison between DBD and DCD samples at pre-transplant was performed. An OPLS-DA model was built with training dataset (DBD n=30, DCD n=5), and the model was tested with the test dataset (DBD n=5, DCD n=7). As shown in the misclassification table, test samples in DBD group can be predicted with 100% accuracy, while the DCD samples were predicted with 85.71% accuracy (**Table 4.2**).

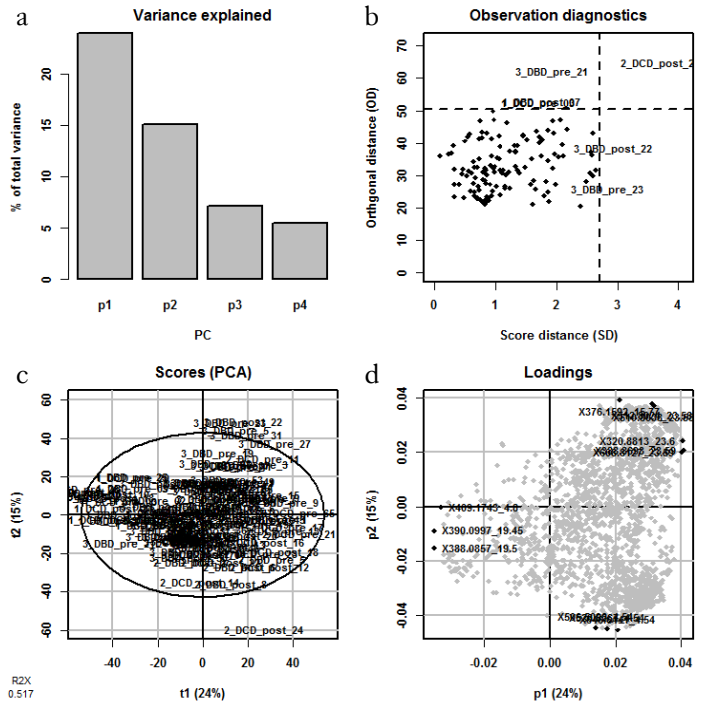


Figure 4.2. PCA summary plots based on 96 samples. a) shows variance explained by each principal component; b) is a diagnostic plot showing the distance of observations orthogonal to the projection plane and the distance of observation projections to the regular/majority data; c) is a score plot, strong outliers can be seen if they are outside the Hotelling's T^2 circle; d) shows a loading plot showing which metabolic features drive PC1 and PC2.

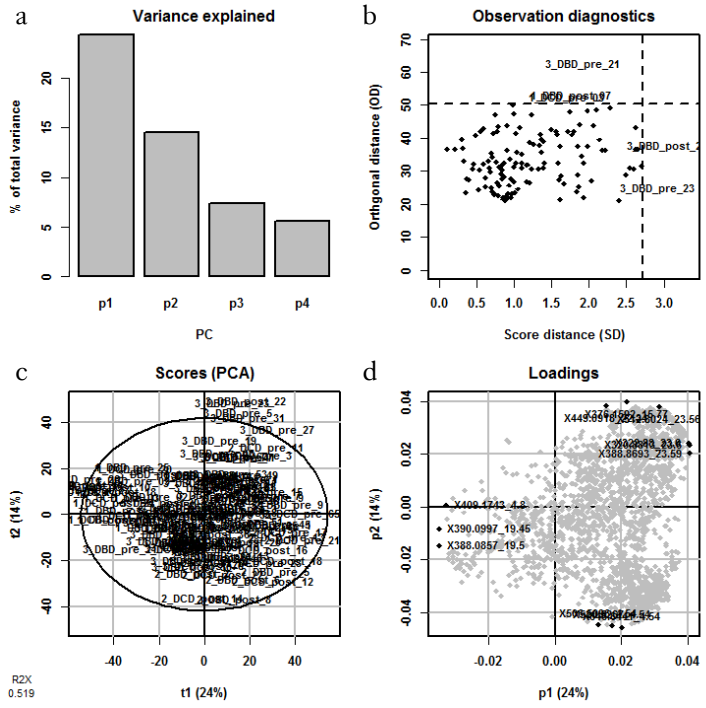


Figure 4.3. PCA summary plots based on 95 samples. a) is a variance plot; b) is a diagnostic plot showing moderate outliers; c) is a score plot; d) shows a loading plot.

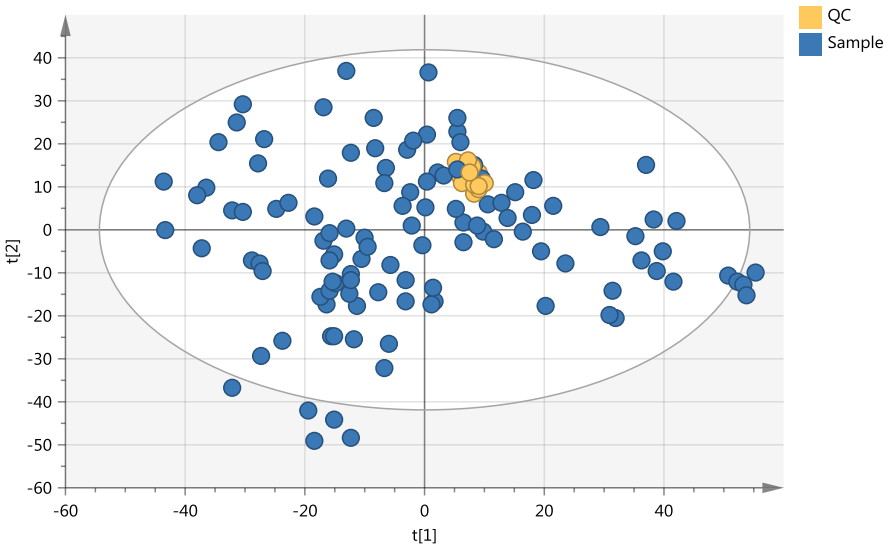


Figure 4.4. PCA plot summarised the distribution of QCs and samples. QCs were represented as yellow circles, and samples were represented as blue circles.

Table 4.2. Misclassification table for the test dataset based on the training dataset model.

		Calculated classes		
		DBD	DCD	Accuracy
True classes	DBD	5	0	100%
	DCD	1	6	85.71%
	Total	12		91.67%
	Fisher's prob.	0.0076		

In order to identify which metabolite features are the strongest discriminators between DBD and DCD at pre- transplant, S-plot (**Figure 4.5**) derived from the OPLS-DA model (R^2X=0.519, R^2Y=0.669, Q^2=0.352) was then studied, 12 features were selected based on the criteria stated in **4.2.4**. From the 12 selected features, a panel of 5 metabolites were annotated as shown in **Table 4.3**.

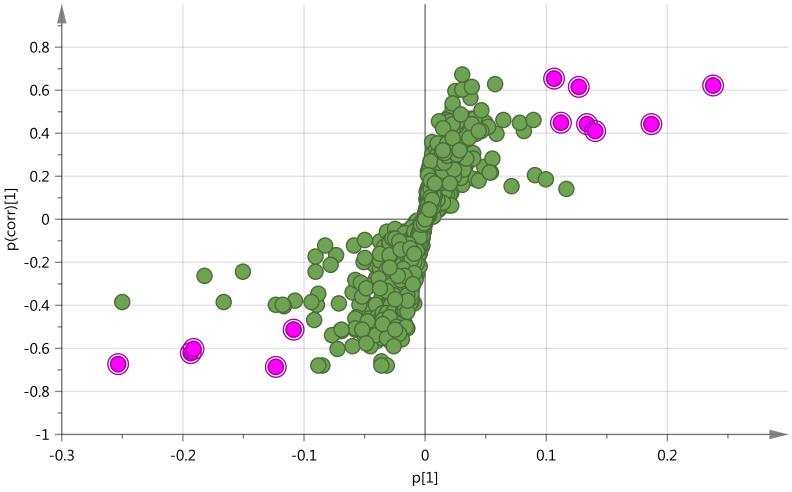


Figure 4.5. Metabolic feature selection from the S-plot. All the dots represent detected features, and the pink dots were selected for annotation.

Table 4.3. Annotation of markers based on molecular weight, retention time and collision induced dissociation fragmentation of 5 metabolites.

Identified as	m/z	Retention time (min)	Observed ion	QC RSD (%)
Adenine monophosphate	348.08	17.3	$[M+H]^+$	12.80
Adenosine	268.10	14.2	$[M+H]^+$	5.88
Adenine	136.06	17.1	$[M+H]^+$	7.16
Hypoxanthine	137.05	9.75	$[M+H]^+$	6.07
Uric acid	169.04	11.8	$[M+H]^+$	21.33

4.3.3 Relative amount of the identified 5 features across groups

From the previous section, 5 discriminating features between DBD and DCD at pre-transplant stage were selected and annotated. To compare how the relative amounts of

5 metabolites vary across all eight groups, jittered scatterplots were produced as shown in **Figure 4.6**.

At pre-transplant stage, the level of AMP and adenine were significantly higher in the DBD group compared to DCD (q<0.01 and q<0.001). Contrastingly, it is observed that uric acid is more abundant in DCD at the pre-transplant stage compared to DBD (q<0.001). Additionally, the scatter plots illustrated that the amount of AMP and adenine were higher in the IGF group compared to EAD. The Mann-Whitney test confirmed that the mean levels of adenine and AMP were significantly statistically different between EAD and IGF (q<0.05). On the other hand, adenosine, hypoxanthine and uric acid showed no significant difference in the level distribution between EAD and IGF groups.

At the post-transplant stage, the Mann-Whitney test revealed that the level of AMP, adenine (q<0.01) and Hypoxanthine (q<0.05) were significantly higher in the DBD group compared to DCD. On top of this, the scatter plot in **Figure 4.6d** and **Figure 4.6e** illustrated that the amount of hypoxanthine and uric acid were more abundant in the EAD group compared to IGF with statistical significance (q<0.05). However, interestingly, the abundance of hypoxanthine in all four groups is relatively lower compared to the other metabolites at post-transplantation.

4.3.4 ROC analysis and clinical correlation

The prediction ability of purine metabolites and general liver function indicators at pre-transplant were evaluated with ROC analysis. The accuracy, area under the curve (AUC), sensitivity and specificity for individual metabolite and enzyme in predicting IGF were listed in **Table 4.5**. Adenine and AMP both showed reliable prediction ability with high AUC and specificity value.

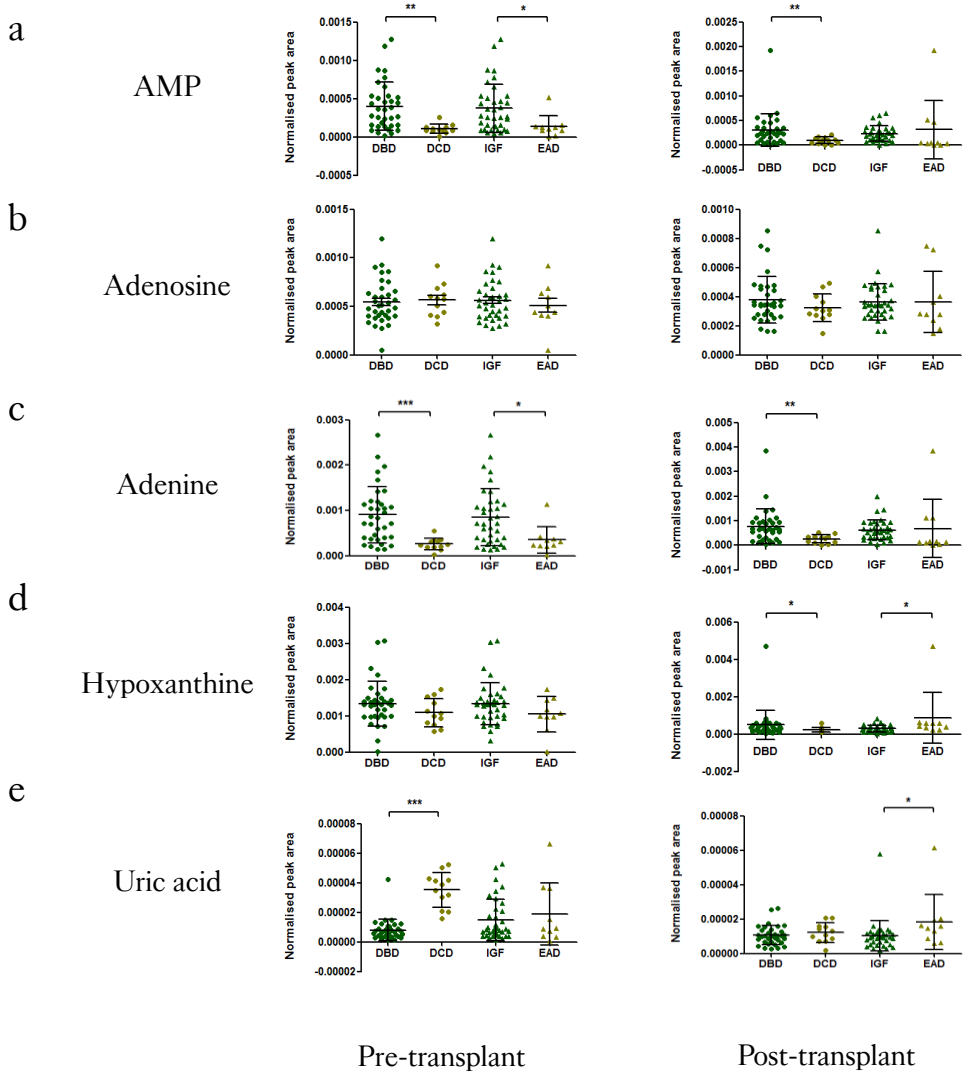


Figure 4.6. Jittered scatter plots of 5 metabolites in four groups at both transplant stages. a) AMP, b) Adenosine, c) Adenine, d) Hypoxanthine and e) Uris acid. AMP, adenosine monophosphate; DBD, donation after brain death; DCD, donation after circulatory death; IGF, immediate graft function; EAD, early allograft disfunction. Results represented as mean ± SD, p-value was derived from Mann-Whitney test, followed by Benjamini-Hochberg FDR correction (*q<0.05, **q<0.01, ***p<0.001).

In order to investigate whether liver enzymes had any effects on these metabolic features, partial correlation analysis was employed. Five metabolites relative amount in pre- and post-transplant samples, together with AST, Bilirubin and GGT in donors on the day of operation (day 0) and in recipients on the day after operation (day 1) were included for correlation analyses. In **Table 4.5**, the only significant correlation was observed between hypoxanthine and bilirubin after Bonferroni correction.

Table 4.4. ROC analysis for 5 annotated metabolites and 3 liver enzymes at pre-transplant for the prediction of IGF.

	AUC	Accuracy	Sensitivity	Specificity
Adenine	0.74	0.52	0.45	0.78
AMP	0.72	0.51	0.44	0.78
Bilirubin	0.64	0.62	0.65	0.50
GGT	0.61	0.62	0.71	0.28
Adenosine	0.60	0.53	0.54	0.50
AST	0.57	0.68	0.76	0.36
Hypoxanthine	0.54	0.59	0.67	0.28
Uric acid	0.53	0.80	0.98	0.07

Table 4.5. Partial correlation analysis (Pearson's correlation with adjusting for patient age) between the 5 selected metabolites and liver enzymes. *p*-values were represented as *q*-values after applying Bonferroni correction. **p*/*q*<0.05, ***p*/*q*<0.01.

Metabolites		AST	Bilirubin	GGT
Adenine	Coefficient	-0.045	-0.122	-0.134
	p-value	0.968	0.321	0.275
	q-value	1	1	1
Adenosine	Coefficient	-0.005	-0.274	-0.084
	p-value	0.967	0.024*	0.496
	q-value	1	0.072	1
AMP	Coefficient	-0.009	-0.097	-0.106
	p-value	0.945	0.430	0.390
	q-value	1	1	1
Hypoxanthine	Coefficient	-0.189	-0.320	-0.039
	p-value	0.122	0.008**	0.752
	q-value	0.366	0.024*	1
Uric acid	Coefficient	0.042	-0.019	0.204
	p-value	0.733	0.875	0.095
	q-value	1	1	0.285

4.4 Discussion

In chapter 2, a lipidomics approach was applied to 112 liver biopsies from DBD and DCD at pre- and post-transplant stages. In this report, hydrophilic metabolic features in 94 liver samples were investigated using HILIC LC-MS technique.

By conducting multivariate PCA, the distribution of outliers and QC samples were examined (**Figure 4.2**, **Figure 4.3** and **Figure 4.4**). One outlier was excluded, and the close cluster of QCs confirmed the good reproducibility of the sample preparation and analysis. Using OPLS-DA built with a training dataset, the accuracy of the model prediction was validated with the test dataset. This training-test approach prevented 'overfitting' and evaluated the model with the classification accuracy. With the test dataset, all the features discriminated DBD from DCD with the accuracy of 91.67%, which implied the reliability of the OPLS-DA model.

The five annotated purines discovered from the OPLS-DA model are generated from the purine metabolism pathway. During energy production, phosphate groups are sequentially hydrolysed from ATP, creating ADP then AMP [421]. From AMP the other metabolites are generated via a number of different catabolic pathways (**Figure 4.7**) [422]. It is understood that the purines are physiological regulators of leucocyte function and inflammation [423], but to be functional they must be released at appropriate loci following stimuli [424]. Inflammation in liver allografts are mainly associated with the DBD transplant type [425]. Previous studies on pre-transplant liver biopsies have shown high levels of pro-inflammatory changes [426, 427]. This may be responsible due to a cascade of inflammatory events that occur in the donors after brain death [428]. On the other hand, studies have shown that DCD tissues undergo less

inflammation but higher rates of hepatocellular damage with increasing warm ischaemia time [428, 429]. Since metabolites in the AMP pathway are well known markers in regulating inflammation [430] and causing oxidative injury, a connection can exist between energy metabolism, inflammation and the extent of ischemic tissue damage [431, 432].

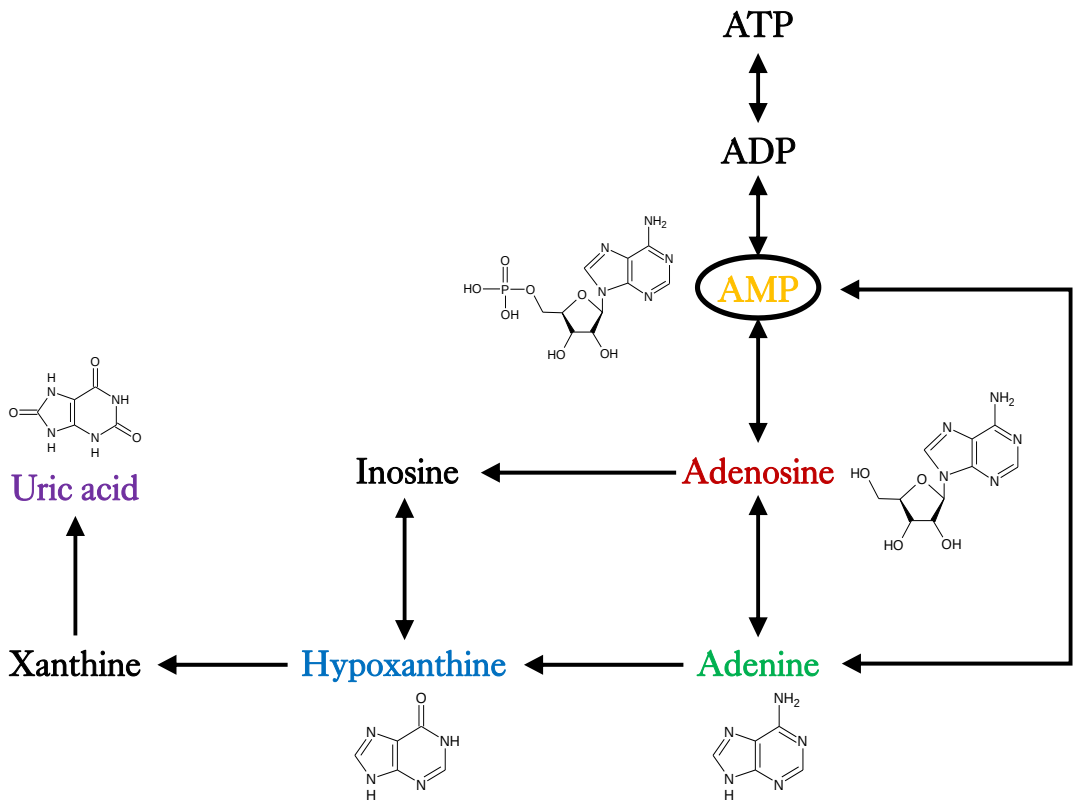


Figure 4.7. Metabolic pathway of AMP, adenosine, adenine, hypoxanthine and uric acid. ATP, adenine triphosphate; ADP, adenine diphosphate; AMP, adenine monophosphate.

AMP is converted from ATP by ectoapyrase (CD39) expressed on the cell surface [433], and is known to be released at the site of vascular injury when platelets aggregate; to promote endothelial barrier function during inflammation [422]. AMP has a role in endothelial resealing during polymorphonuclear leukocyte transendothelial migration

[434]. Michael *et al.* found that gene-targeted mice with overexpression of CD39 conferred protection in both warm and cold hepatic ischaemia [435]. This is in line with high levels of AMP (to serve as a protective mediator during inflammation) observed in DBD (**Figure 4.6a**). It has also been shown that dying cells as a result of ischaemia undergo lysis to release adenine [436]. Kartha *et al.* demonstrated *in vitro* that adenine nucleotides accelerated structural and functional recovery in epithelial cells [437]. This suggests why DBD liver allografts (**Figure 4.6b**) with elevated levels of adenine could speed up recovery time.

To understand the potential applicability of adenine and AMP to transplantation, clinical outcome groups were investigated. The results found higher levels of adenine and AMP in IGF versus EAD (q<0.05) (**Figure 4.6c)**. Studies have shown that adenine and AMP have a protective function during inflammation [434]. The elevated presence in IGF group also suggests that both adenine and AMP could be studied further as markers to predict primary graft function. In addition to being mediators for graft recovery, Roy *et al*. found that having high levels of AMP during strenuous conditions such as during ischaemia when oxygen is low, indicates ATP is still being generated [438]. One molecule of ADP phosphorylates another molecule of ADP to form ATP and AMP [438, 439]. During these conditions for every one molecule of ATP, six molecules of AMP are generated [439]. This might explain why DBD allografts and IGF groups show an increased level in AMP, as there may be continued energy metabolism improving primary graft function [440]. The prediction model in regard to IGF revealed that the diagnostic potential of AMP and adenine. Higher AUC and specificity values were observed when compared to traditional enzyme makers, which showed moderately lower accuracy.

Furthermore, Wyatt *et al.* found that arresting hearts with a cardioplegic solution containing hypoxanthine maintained ATP content during I/R in dogs, and enhanced functional recovery during the post-ischemic period [441]. This explains the observed elevated level of hypoxanthine in DBD post-transplant stage (**Figure 4.6d**). In addition, higher amount of hypoxanthine was observed in EAD compared with IGF (q<0.05). Interestingly, Heshmatollah *et al.* in a cohort of 30 patients, observed that 29.4% of them who demonstrated EAD had also elevated hypoxanthine levels [442]. This suggests that hypoxanthine generated during ischaemia may have a link with EAD however further investigation is needed to support this. Although no obvious association was observed between hypoxanthine and bilirubin in hyperoxia rat brain [443], correlation analysis performed in this study revealed negative correlation between these two molecules. However, the clinical evidence to support this observation is relatively weak.

Increased level of uric acid was observed in DCD patients at pre-transplant (q< 0.001) (**Figure 4.6e**). DCD allografts are prone to IRI due to being exposed to an extensive period of warm ischaemia [444]. In humans, uric acid is the final product of purine metabolism [445]. The role of uric acid in conditions associated with oxidative stress is not entirely clear. However, epidemiological studies suggest that during ischaemia reperfusion injury, there are increased levels of uric acid which make it has potential as an oxidative stress marker [446]. A similar observation was made in a study conducted by Matthew et al., in which hepatic ischaemia was induced for 30 min followed by 60 min of reperfusion. After ischaemia, uric acid levels had increased by over 300% and during the first 30 min of reperfusion by 600% [447]. In addition, the appearance of EAD could act as a valid predictor of both graft failure and death in DBD allograft recipients, whereas there is no obvious difference between DCD allograft recipients with

or without EAD in terms of the rate of graft failure or death [345]. In this investigation, a significant difference in uric acid was not observed at pre-transplant between IGF and EAD, and a moderate elevation was seen in EAD ($q < 0.05$) post transplantation (**Figure 4.6e**). Although no significant difference was seen in uric acid between DBD and DCD in post-transplantation, it could be related to redistribution of uric acid after reperfusion.

Although no significant differences were seen between DBD and DCD at both transplantation stages (**Figure 4.6**b), adenosine has recently been discussed widely as a target for cancer immunotherapy [448]. Adenosine and ATP are generally present at very low levels in extracellular fluids [449]. Inflammation, ischemia, or cancer can lead to the release of high levels of ATP, which consequently stimulate the formation of AMP and adenosine [450]. In contrast to the pro-immune properties of extracellular ATP, extracellular adenosine can dampen the immune response through restraining effector cell function and stabilizing regulatory cell immunosuppression [451].

To understand results further, additional enzymes related with the purine pathway in human liver could have been measured, to understand the reasons for why particular metabolites demonstrated elevated/diminished levels.

4.5 Conclusion

In this study, the analysis of polar metabolites in the context of liver transplantation has identified five polar metabolites differentiated in DBD and DCD livers. Adenine and AMP showed higher levels in DBD group and were also elevated in recipients that experience IGF at pre-transplant. Other relevant molecules such as uric acid and hypoxanthine showed a moderate association to IRI. This study has been relevant in identifying pre-transplant molecules that could be related to better outcomes and tissue quality for liver transplantation.

Chapter 5 A lipidomics approach to the cholestasis mouse model

The named researchers below contributed to this chapter by conducting the following experiments.

1. Dr Khadija Alawi (King's College London, UK): diet induction and sample collection.
2. Mr David Tandio (King's College London, UK): sample collection.
3. Mr Pratish Thakore (King's College London, UK): mice breeding and cholestasis assessment assays.

5.1 Introduction

Cholestatic liver diseases consist of primary biliary cirrhosis, familial intrahepatic cholestasis and intrahepatic cholestasis of pregnancy, resulting in principle and systemic pathology, and critically, liver failure [452]. These pathologies are characterised by the impairment of hepatocellular and/or cholangiocellular secretory function and bile flow, leading to elevation of hepatic and serum bile acid content [453]. During cholestatic liver diseases, aggregation of bile acids not only disrupts bile acid homeostasis directly, but also affects subsequent retention of lipids in the liver, ultimately preceding to hepatocyte injury and inflammation [454].

Bile is a complex biofluid produced by the liver and stored in the gallbladder. The major functional constituents of bile are bile acids (BAs) which are also known as steroid acids [455]. The release of BAs from the gallbladder to small intestine is triggered by the ingestion of food. As a digestive agent, BAs facilitate the absorption of lipids by emulsification as well as other fat-soluble vitamins through formation of micelles, which play an important role in regulating cholesterol homeostasis [456].

The production of bile acids mainly occurs in hepatocytes. There are two pathways responsible for the synthesis of primary BAs [457]. The classic (or neutral) pathway, which is the major biosynthetic pathway in human, contributes about 90% of the total BAs synthesis in the liver. The alternative pathway, which is also named acidic pathway due to the generation of acidic intermediates, produces less than 10% of the total BAs under routine biological conditions [458]. Two primary BAs, chenodeoxycholic acid (CDCA) and cholic acid (CA) are synthesized from cholesterol through the classic pathway while only CDCA is produced under the acidic pathway [459]. There are three

other primary bile acids, named α-muricholic acid (αMCA), β-muricholic acid (βMCA), and Hyocholic acid, which are transformed from CDCA. In the liver, CDCA and CA are conjugated with two amino acids, glycine and taurine, to synthesize glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA), and taurocholic acid (TCA). In the intestine, GCDCA, TCDCA, GCA and TCA yield glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), glycolithocholic acid (GLCA) and taurolithocholic acid (TLCA) through bacterial dehydroxylation while CDCA undergoes epimerisation producing glycoursodeoxycholic acid (GUDCA) and tauroursodeoxycholic acid (TUDCA) [355]. When these bile acids are secreted into the lumen of the intestine, bacterial deconjugation and removal of the glycine and taurine groups produces the secondary bile acids, deoxycholic acid (DCA), lithocholic acid (LCA), hyodeoxycholic acid (HDCA) and ω-muricholic acid (ωMCA) [460]. These bile acids cycle through enterohepatic circulation where they can be taken back up into the blood stream, return to the liver, and be re-secreted to bile. Moreover, a fraction of CDCA is also transformed into tertiary bile acid, ursodeoxycholic acid (UDCA), in the liver. The illustration of bile acids synthesis can be seen in **Figure 5.1**.

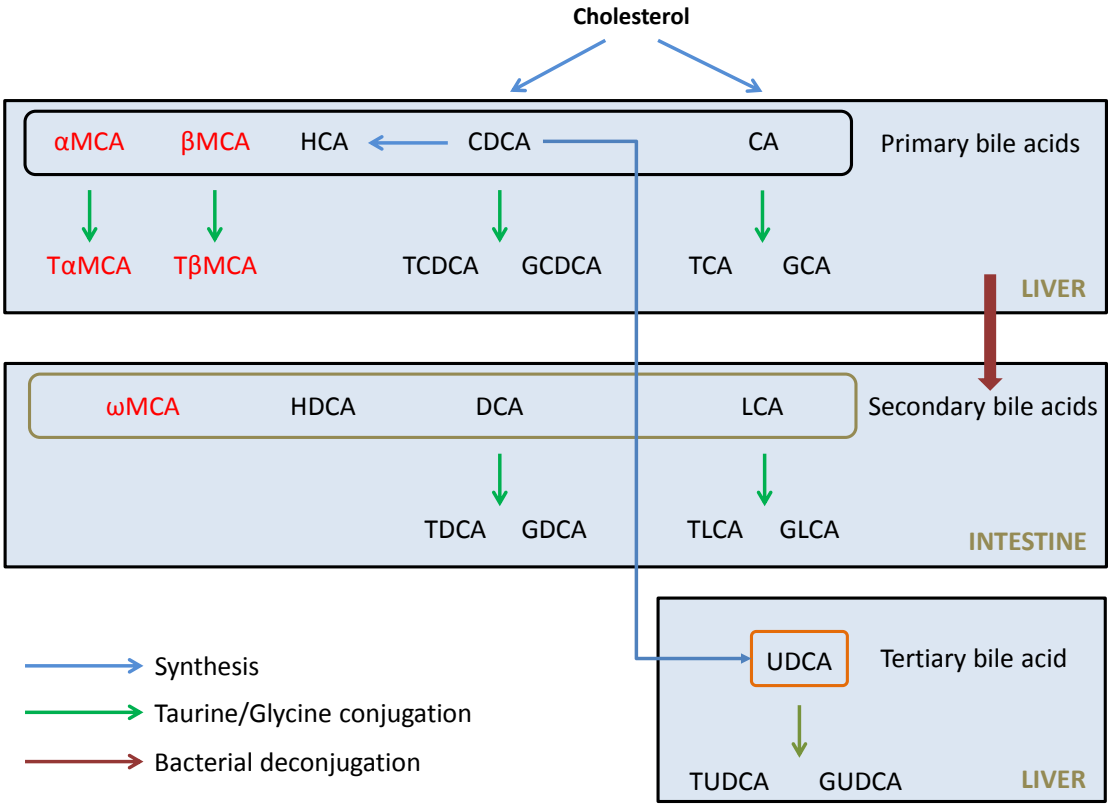


Figure 5.1. Bile acids synthesis in mammals. The synthesis, conjugation and deconjugation of 22 bile acids in mammals. Color red represents bile acids only exist in mice, not in human.

Cholic acid is elevated in patients with liver disease, and rodent experimental models of cholestasis employing exogenous CA, e.g. via dietary supplementation, precedes hypercholanemia, dyslipidaemia and cholestatic symptoms [461]. Despite the increased knowledge of bile acid signaling and homeostasis, through identification of bile acid receptors, the pathogenesis of cholestasis remains unclear, with limited drug treatments available. While increased serum bile acids and plasma ALP and GGT remain diagnostic markers for cholestatic hepatic disease, there is expanding evidence for dysregulation of lipid homeostasis, resulting in an increase in hepatic and plasma lipids/metabolites [453, 461].

5.2 Methods

In this chapter, untargeted lipidomic fingerprints and semi-targeted bile acids profile of liver (from 13 mice, 7 RM3 mice and 6 CA mice) and plasma (from18 mice, 8 RM3 mice and 10 CA mice) were obtained using LC-MS approach. A work flow for this study can been seen below (**Figure 5.2**).

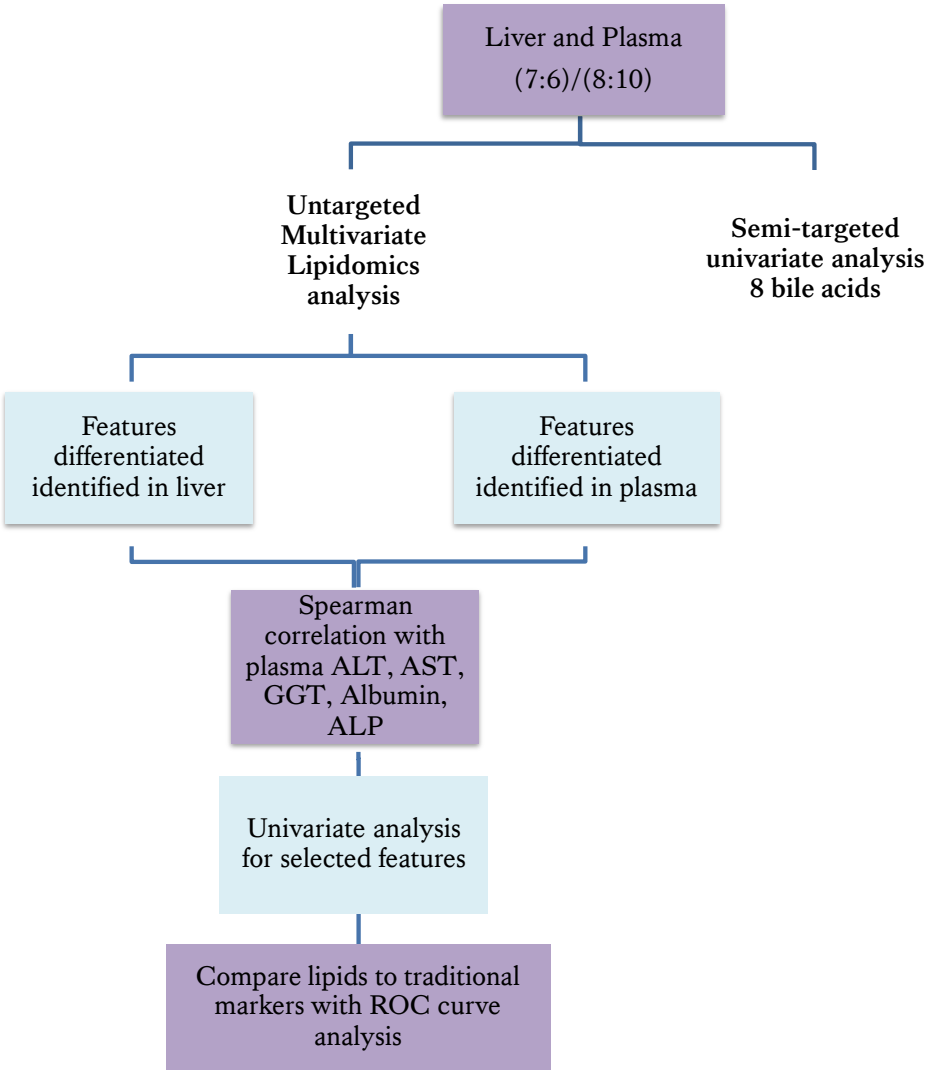


Figure 5.2. Data analysis work flow for mice liver and plasma fingerprints by LC-MS. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; ROC, receiver operating characteristic.

5.2.1 Reagents

Solvents used for LC-MS analysis including water, methanol and methyl tertiary butyl ether (MTBE) were LC-MS grade purchased from Sigma-Aldrich (Poole, UK). Ammonium formate and two internal standards, prednisolone (≥ 98% purity) and triglyceride (45:0) (≥ 98% purity), were purchased from Sigma-Aldrich. All other drugs and reagents were from Sigma-Aldrich (Dorset, U.K.) unless otherwise stated.

5.2.2 Mice

Animals were housed under temperature (22±2˚C)-controlled colony rooms maintained under filtered positive pressure ventilation on a 12-12 h light/dark cycle beginning at 07:00 GMT with free access to water and food. Male, 129S1/SvImJ wildtype (WT) littermates were used at 8-12 weeks of age. All experiments were conducted under the guidelines of the United Kingdom Home Office Animals (Scientific Procedures) Act 1986 and were approved by the King's College London Animal Care and Ethics Committees. All experiments were conducted in a blinded manner. Animals were randomly assigned to control or treatment groups.

5.2.3 Dietary induction of cholestasis

Mice were randomly allocated to receive standard animal maintenance diet (RM3, Special Diet Services, UK) or supplemented with 0.5% CA (0.5% CA in RM3 diet, Special Diet Services, UK), for 21 days [461]. Food and water consumption, and body

weight were recorded every other day for the duration of the experiment. For 18 mice in total, 8 were classified for CA diet, and 10 were allocated for RM3 diet for control purpose.

5.2.4 Plasma/liver biochemistry

Mice were anaesthetised with isoflurane (2-3% carried in 2-3% O_2) and blood samples were obtained via a cardiac puncture, using a heparinised syringe and needle (100 U/ml). Plasma was separated with centrifugation (400×*g*, 20 min), snap frozen in liquid nitrogen and stored at –80˚C until processing. Whole liver was weighed and snap frozen in liquid nitrogen for further analysis. Plasma albumin, alanine transaminase (ALT), alkaline phosphate (ALP), aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT) levels were measured according to manufacturer's instructions.

5.2.5 Bile acids and lipids extraction

To extract bile acids from the liver tissue, between 50 and 80 mg of tissue were obtained and transferred to a pre-weighed 2ml Eppendorf tube containing a steel bead (5mm, Qiagen, MD, US). Then, 20 µL (per 1 mg of tissue) of methanol (containing the negative-mode internal standard prednisolone, 10µg/mL) was added, and samples were homogenized for 3 min at 25 Hz (6 cycles of 0.5 min) in a TissueLyser (Qiagen, MD, US). Following this, samples were centrifuged with 12,000×*g* for 20 min at 4 ºC (Centrifuge 5810R, Eppendorf, Germany). Then, 600 µL of the supernatant was

transferred to continue with the solid phase extraction (SPE) steps with a C18 cartridge (Bond Elut C18, Agilent, UK). Quality control samples were created by pooling excess supernatant from each biological sample to follow the same SPE steps [462].

For the extraction of the bile acids from plasma, 150 µL of methanol (containing the negative-mode internal standard prednisolone, 10µg/mL) was added to 50 µL of plasma, and vortex for 30 s. Samples were then incubated at -20 ºC for 20 min, and centrifuged with 12,000×*g* for 10 min. The supernatant was transferred to a clean vial and dried with nitrogen evaporator (REACTI-THERM III, Thermo Scientific, US) at 37 ºC. The dried residual was then reconstituted in 50 µL of methanol [355, 463]. Quality control samples were taken from the pool of all biological sample (10 µL from each).

Lipidomics analysis for liver tissue was performed as described in **Chapter 2.2.5**. To extract lipids from the plasma, samples were thaw on ice and vortexed for 1 min. 20 µL of plasma was then mixed with 10 µL of water, 40 µL of methanol and vortexed for 2 min (VX-2500 Multi-Tube Vortexer, VWR, US). Following this, 200 µL of MTBE containing 10µg/mL of internal standard (triglyceride (45:0)) was added, and vortexed at room temperature for 1 hour. After cooling, 50 µL of water was added, and samples were centrifuged at 4,000×*g* for 10 minutes. 50 µL of the upper MTBE layer was then transferred to a clean HPLC vial (with 300 µL insert) for each sample while 10 µL of the MTBE layer was collected for pooled QC from all samples.

5.2.6 Chromatographic and mass spectrometric conditions

All extracted samples were stored at -80 °C before injection. Details of chromatographic separation for lipids were provided previously (see **Chapter 2.2.5**). Separation of bile acids was achieved by using an Supelco Titan ™ UHPLC C18 column (100mm × 2.1mm, 1.9 µm), maintained at 40 ˚C. A gradient was employed consisting of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The solvent was delivered at a flow rate of 0.5 mL/min. Gradient analysis start with 60% mobile phase A and 40% mobile phase B for the first 4 min. Then increase to 100 % mobile phase B at 10 min, and maintained for 2 minutes with 100% mobile phase B. At 12.1 min, the composition changed back to 60% mobile phase A and 40% mobile phase B and maintained till 15min.

Analysis of the extracted samples was performed using Waters ACQUITY ultra-performance liquid chromatography-quadrupole time of flight (UPLC-QToF) in positive ionisation mode for lipids and negative ionisation mode for bile acids [326]. For positive mode, a capillary voltage of 3.2 kV and a cone voltage of 45 V were used. The desolvation gas flow was 400 L/hour and the source temperature was 120˚C. For negative mode, a capillary voltage of -2.7 kV and a cone voltage of 50 V were used. Desolvation gas flow and source temperature were fixed at 600 L/h and 300 °C, respectively. All analyses were acquired using the lock spray to ensure accuracy and reproducibility, a reference solution leucine enkephalin was used as lock mass (m/z 556.2771, 278.1141 for positive and 554.2615, 263.1035 for negative mode) at a concentration of 500 ng/mL and a flow rate of 10 µl /min to infuse at regular intervals.

Data were collected in the centroid mode over the mass range m/z 50–1000 with an acquisition time of 0.1 second per scan.

5.2.7 Data processing and analysis

Lipidomics data processing was carried out within 'XCMS' package in 'R Studio' (version 1.0.153). For the multivariate analysis, OPLS-DA models were built in SIMCA (version 14, MKS Umetrics AB, Sweden) to compare control and CA-fed mice. Feature selection was done using thresholds of Variable Importance for the Projection (VIP) score >1, loading score >0.05 and coefficient value >0.01, common features from these three platforms were chosen for further analysis. The identification (level 1) was performed by comparing structure and fragmentation patterns in the MS^2 data with standards or in-house database.

All identified bile acids and annotated lipids were measured in the LC-MS data using Waters MassLynx software (Waters Corporation, Milford, MA) and peak areas were normalized to internal standard.

Permutation test was conducted based on the built OPLS-DA model in SIMCA. Correlation test between annotated lipids and plasma enzymes for both liver and plasma matrixes were performed in 'R Studio' (version 1.0.153). Mann-Whitney test followed by Benjamini-Hochberg correction was carried out to compare the selected lipid levels between cholestatic and control groups (bar plot generated from GraphPad Prism 6, GraphPad, US). Receiver operating characteristic (ROC) curve was plotted to compare

the prediction ability of bile acids, selected lipids and plasma enzymes for cholestasis in R studio.

5.3 Results

5.3.1 Liver and plasma biochemistry outcomes

With the purpose of evaluating liver injury caused by CA supplementation, mice liver weight, body weight and concentrations of liver enzymes (ALT, ALP, AST and GGT) were measured.

Increased levels of plasma albumin, ALT, ALP, AST, GGT and liver weight/body weight ratio were observed in CA-fed mice. Mann–Whitney U test was applied to examine the statistical differences between two groups. Results are presented as mean ± SD (**Figure 5.3**).

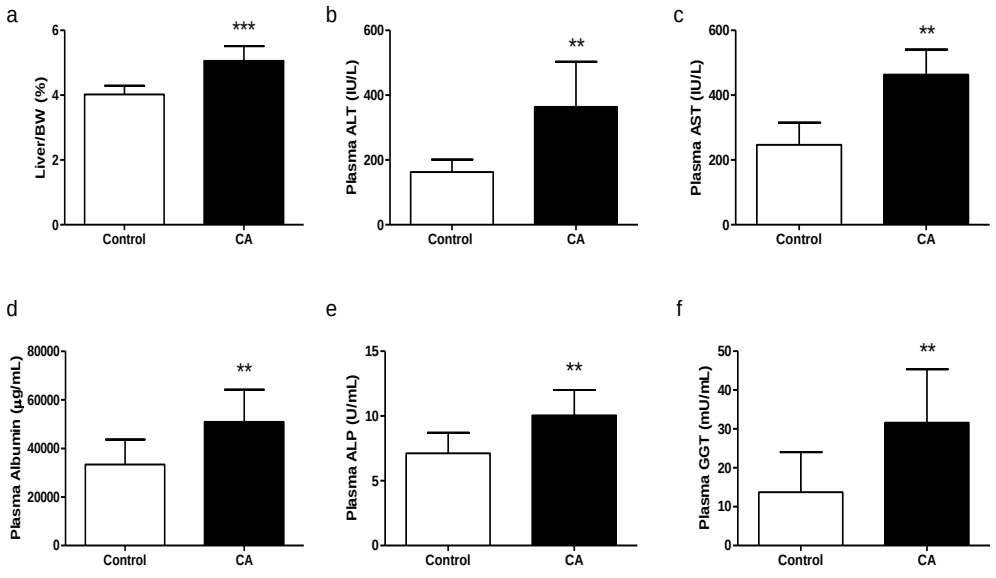


Figure 5.3. Bar plots showing (a) Liver/BW ratios, the concentration of plasma (b) ALT, (c) AST, (d) Albumin, (e) ALP and (f) GGT differentiating between control and CA groups. *p*-value was derived from Mann-Whitney test (**p<0.01, ***p<0.001). CA, cholic acid; BW, body weight; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase.

5.3.2 Bile acids

After conducting a trial with pooled QC samples and 15 bile acid standards, targeted bile acid analysis featured the identification and semi-quantification of 8 bile acids, including CA, TCA, GCA, TCDCA, DCA, TDCA, GDCA, and TUDCA. Standards for individual compound were tested under the same LC-MS conditions as samples. The identification parameters for each standard can be seen in **Table 5.1**. Comparison of individual bile acid and the sum of 8 bile acid contents between control and CA groups in both liver and plasma revealed the accumulation of bile acids in CA fed mice (**Figure 5.4**).

Table 5.1. Bile acids standards identification parameters.

Bile Acids	Retention Time (min)	Mass to charge ratio
CA	3.00	407.28
TCA	0.74	514.28
GCA	1.39	464.30
TCDCA	1.22	498.29
DCA	6.29	391.28
TDCA	1.38	498.30
GDCA	3.90	448.30
TUDCA	0.77	498.30

CA, cholic acid; TCA, taurocholic acid; GCA, glycocholic acid; TCDCA, taurochenodeoxycholic acid; DCA, deoxycholic acid; TDCA, taurodeoxycholic acid; GDCA, glycodeoxycholic acid; TUDCA, tauroursodeoxycholic acid.

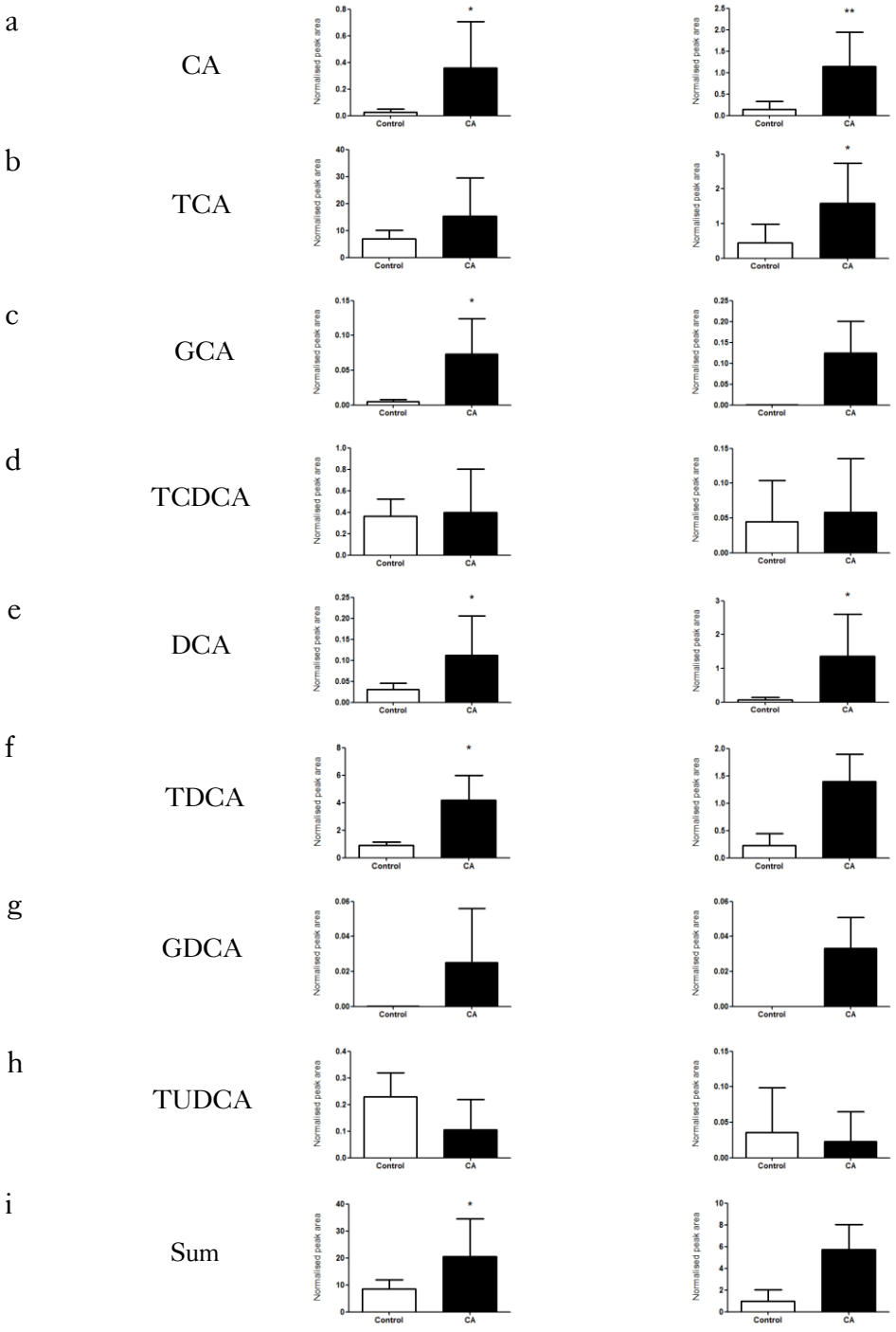


Figure 5.4. Bile acid levels in liver and plasma. Results were expressed as mean ± SD, p-value was derived from Mann-Whitney test (*p<0.05, **p<0.01). CA, cholic acid; TCA, taurocholic acid; GCA, glycocholic acid; TCDCA, taurochenodeoxycholic acid; DCA, deoxycholic acid; TDCA, taurodeoxycholic acid; GDCA, glycodeoxycholic acid; TUDCA, tauroursodeoxycholic acid.

5.3.3 Lipidomics

In order to determine the changes in lipids associated with cholestasis, LC-MS based lipidomics profiling was employed on liver and plasma samples from RM3 and CA fed mice. More than 5,000 lipid features were obtained in lipidomic profiling, and the principal component analysis (PCA) was applied to all samples including QCs. From the unsupervised models in **Figure 5.5**, no outlier was observed in both score plots.

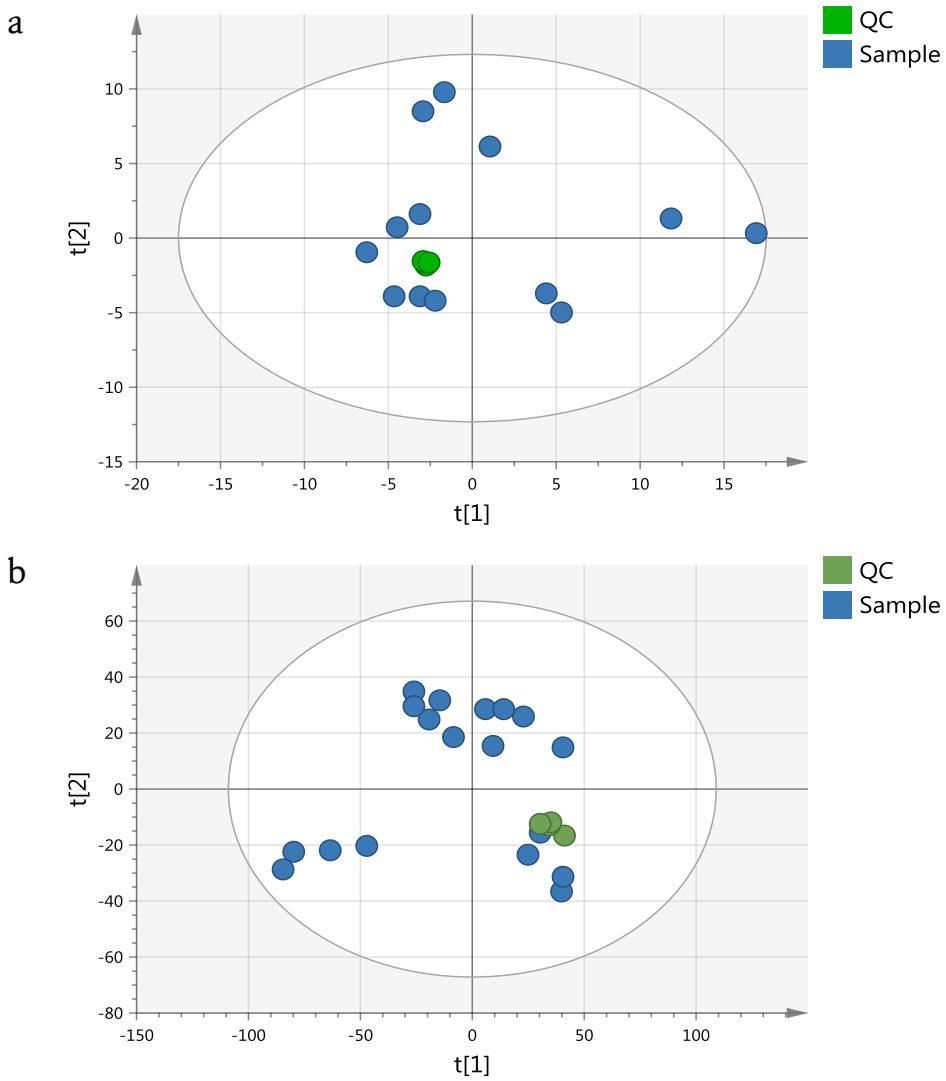


Figure 5.5. PCA score plot of (a) liver lipidomics showing the distribution of 4 QCs and 13 samples (R^2 X=0.73, Q^2=0.61), and (b) plasma lipidomics showing the distribution of 4 QCs and 18 samples (R^2 X=0.5, Q^2=0.378).

After excluding the QCs, the processed data from the LC-MS experiment underwent supervised discriminant analysis by building orthogonal projections to latent structures-discriminant analysis (OPLS-DA) models with liver and plasma samples for control and CA groups (**Figure 5.6**).

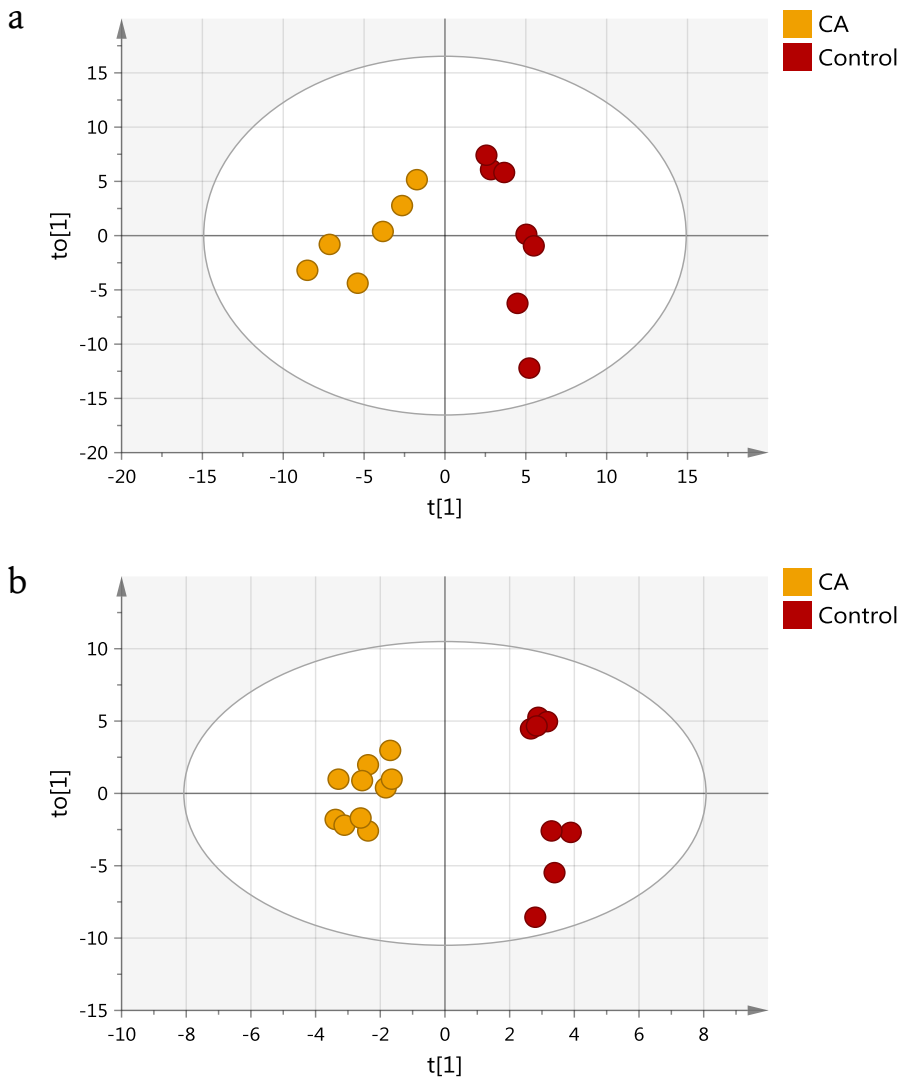


Figure 5.6. OPLS-DA score plot for distinguishing CA fed mice from control mice (a) liver, R^2 X=0.764, R^2 Y=0.861, Q^2=0.803 and (b) plasma, R^2 X=0.502, R^2 Y=0.967, Q^2=0.885.

In the cholestasis model, multivariate analysis was conducted base on a limited number of mice. In order to assess the risk that the built OPLS-DA models could be overfitted, permutation plots (**Figure 5.7**) were included to compare the goodness of fit (R^2 and Q^2) of the original models (far to the right) with those of 20 models (further to the left) where the order of group information has been randomly permutated.

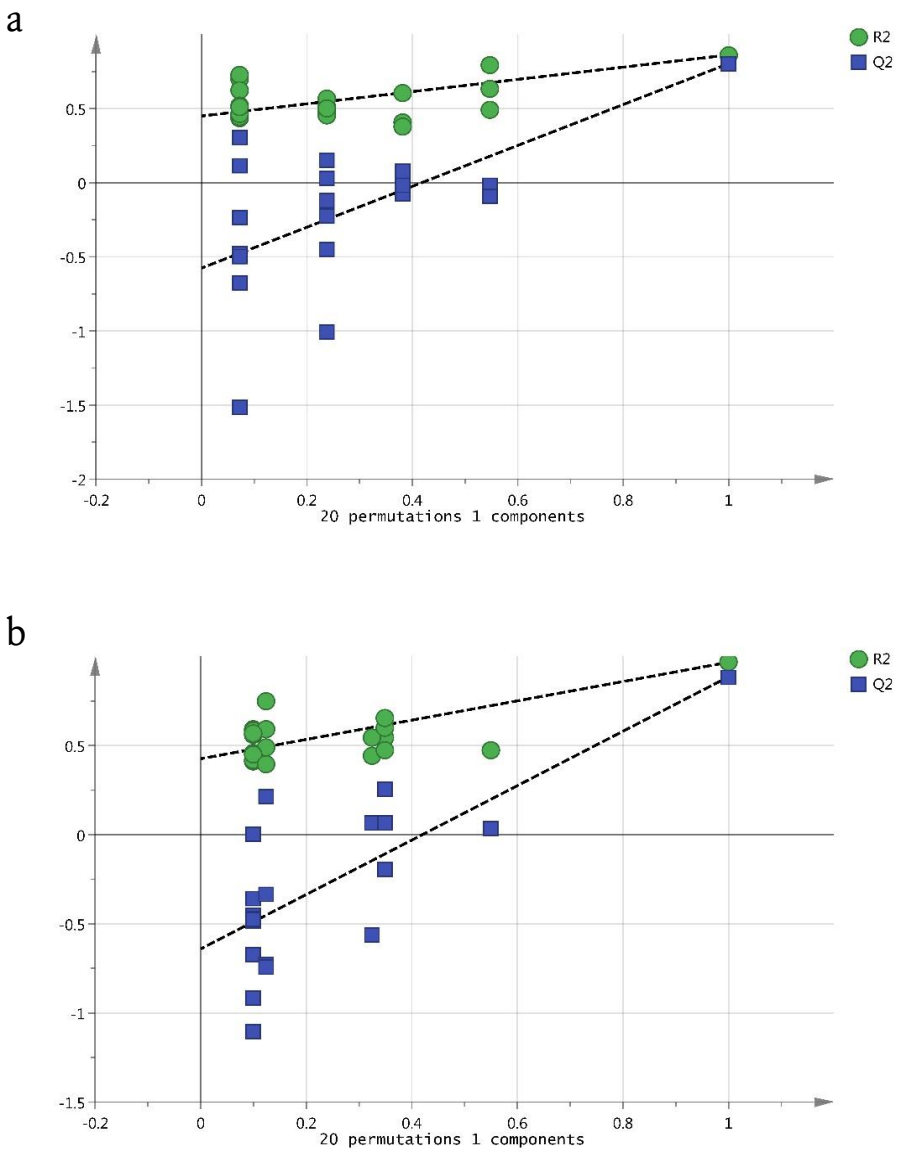


Figure 5.7. Permutation plots for OPLS-DA models. (a)liver and (b) plasma.

In OPLS-DA analysis, apparent separation was observed between control and CA groups suggesting the metabolic change between them was due to the effect of CA supplementation. Through the comparison in both **Figure 5.7a** and **Figure 5.7b**, all blue Q2-values to the left are lower than the original points to the right, on top of that, the blue regression line of the Q2 points interacted with the vertical axis below zero. Permutation tests revealed that the built OPLS-DA models were valid and reliable. 15 metabolic features which would discriminate control from cholestatic mice were annotated in liver, while the plasma model was interpreted by 13 metabolites.

5.3.4 Statistical analysis

Selected features from OPLS-DA models were annotated through online databases and in-house databases. The list of annotated metabolites was included in **Table 5.**. From the annotated metabolites, the correlation between the selected 15 lipids (in liver)/ 13 lipids (in plasma) and 5 plasma enzymes (ALT, AST, GGT, Albumin, ALP) was investigated separately by spearman test.

Table 5.2. Annotated metabolites from OPLS-DA models in liver and plasma.

	Metabolites	Retention time (min)	mass to charge ratio (m/z)
Liver	TG (52:4)	28.91	872.77
	TG (50:1)	31.42	850.79
	PC (32:1)	15.79	732.55
	TG (52:2)	32.42	876.81
	CE (18:3)	26.81	664.61
Plasma	PC (36:3)	17.02	784.59
	PC (34:2)	16.37	758.58
	PC (36:2)	18.07	786.61
	TG (50:2)	29.77	848.77
	TG (52:3)	30.51	874.79
	PC (36:4)	16.49	782.58
	CE (18:1)	29.67	668.64
	CE (18:2)	28.06	666.62
	CE (16:1)	27.47	640.61
	CE (16:0)	28.92	642.62
Liver & Plasma	TG (50:2)	29.79	848.78
	PC (36:1)	18.94	788.62
	LysoPC18	4.95	524.37

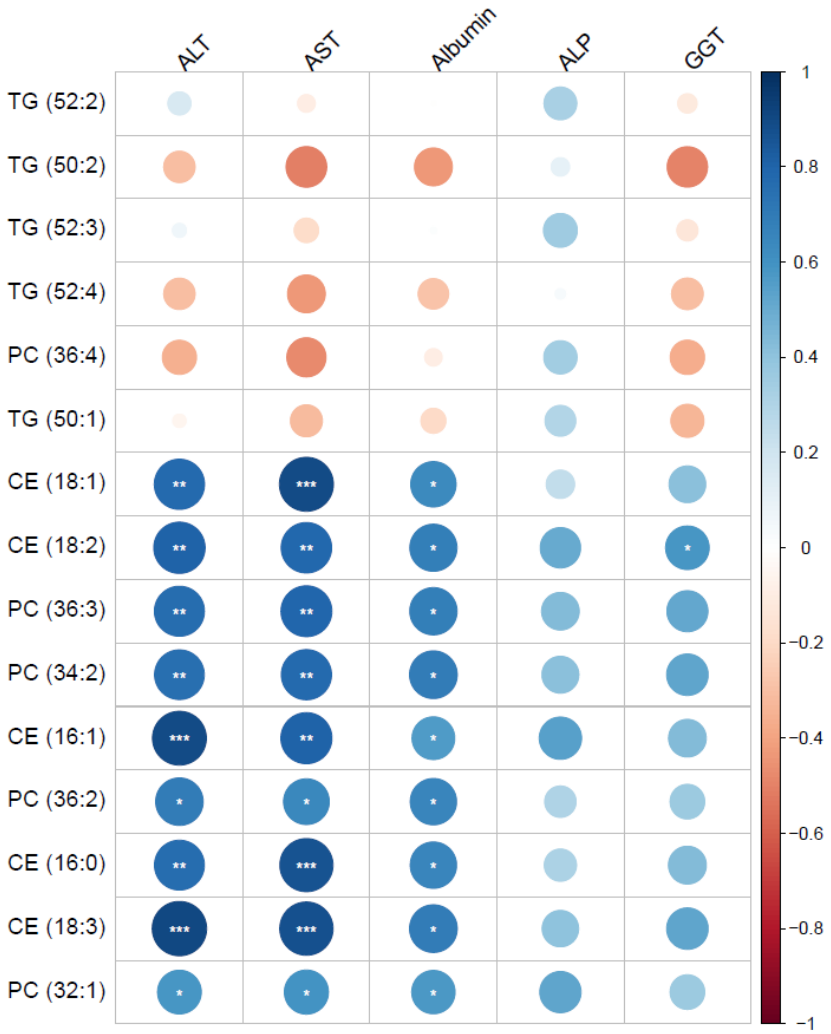


Figure 5.8. Correlation plot of 15 lipids in liver and 5 plasma enzymes. Blue represents positive correlation while red represents negative correlation. Intensity of 'redness' and 'blueness' represents correlation coefficient value. *p<0.05, **p<0.01, ***p<0.001. TG, triglyceride; PC, phosphatidylcholine; CE cholesterol ester; ALT, alanine transaminase; AST, aspartate aminotransferase; ALP, alkaline phosphate; GGT, gamma-glutamyl transferase.

As shown in **Figure 5.8**, CE (18:1), CE (18:2), PC (36:3), PC (34:2), CE (16:1), CE (16:0) and CE (18:3) in liver were significantly positively correlated with ALT and AST (p<0.01). Furthermore, it is observed that CE (18:1), CE (16:0) in plasma positively associating with ALT and AST, while PC (36:3), PC (36:2), PC (36:1) and PC (34:2) were negatively associated (**Figure 5.9**).

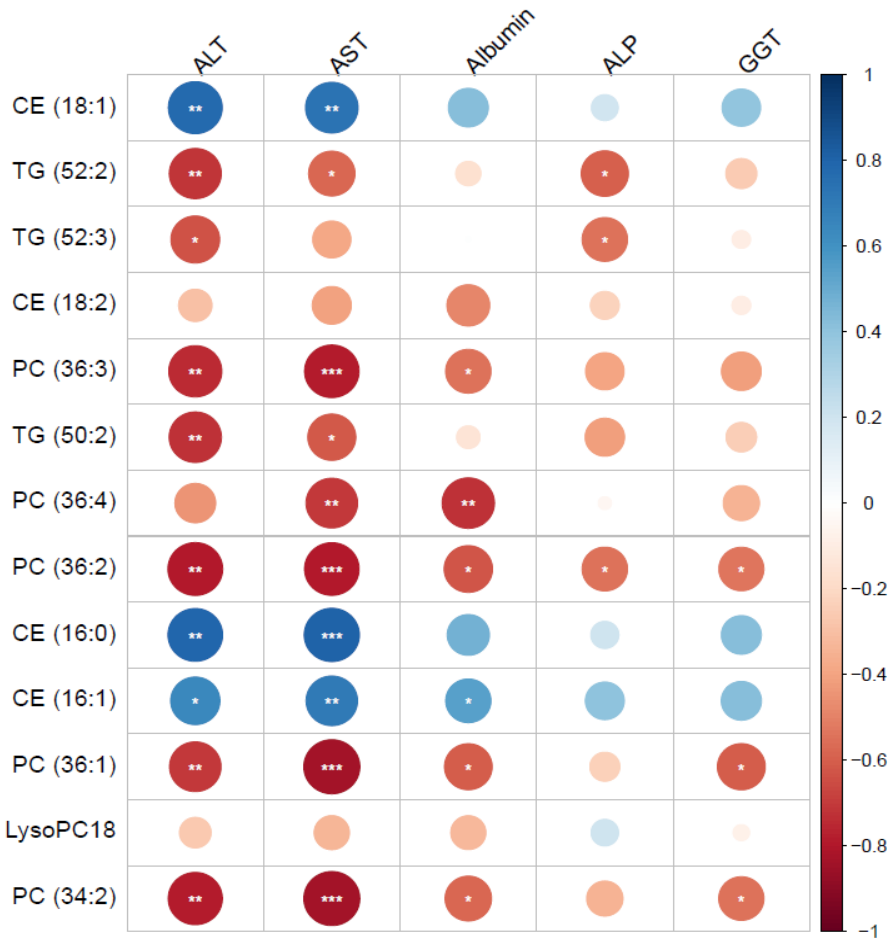


Figure 5.9. Correlation plot of 13 lipids in plasma and 5 plasma enzymes. Blue represents positive correlation while red represents negative correlation. Intensity of 'redness' and 'blueness' represents correlation coefficient value. *p<0.05, **p<0.01, ***p<0.001. CE cholesterol ester; TG, triglyceride; PC, phosphatidylcholine; LysoPC, lysophosphatidylcholine; ALT, alanine transaminase; AST, aspartate aminotransferase; ALP, alkaline phosphate; GGT, gamma-glutamyl transferase.

Univariate analysis was applied for the panel of 15 lipids in liver and 13 lipids in plasma. It discovered 3 lipids putatively identified as cholesterol esters (CE) and 2 phosphocholines (PC) that showed significant differences between control and CA-fed mice in both liver and plasma profiles (**Figure 5.10**). The concentrations of CE (16:0), CE (16:1) and CE (18:1) were significantly higher in CA-fed mice, while levels of PC (36:3) and PC (34:2) showed opposite trend in plasma when compared with liver.

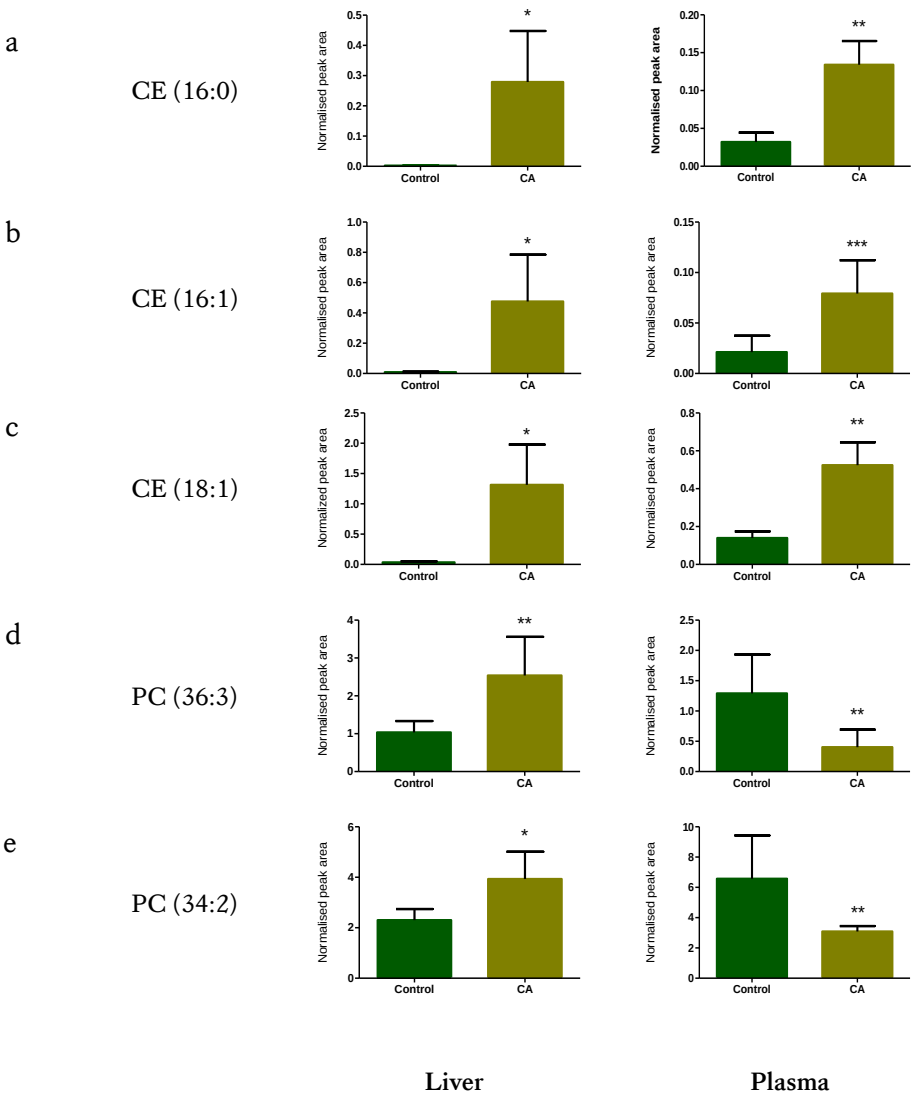


Figure 5.10. Comparison of 5 common lipid contents in liver and plasma between CA-fed and control mice. (a) CE (16:0), (b) CE (16:1), (c) CE (18:1), (d) PC (36:3) and (e) PC (34:2). Results represented as mean ± SD, p-value was derived from Mann-Whitney test followed by Benjamini-Hochberg correction (*p<0.05, **p<0.01, ***p<0.001). CE cholesterol ester; PC, phosphatidylcholine.

The utility of the five selected lipids (3 CEs and 2 PCs) was then tested by comparing with total bile acids (based on 8 targeted analysed bile acids) and plasma enzymes (ALP and GGT) between control and CA-fed mice using area under curve (AUC) metric

from receiver operating characteristic (ROC) curve (**Figure 5.11**). AUC of 77.5%, 100%, 91.2% for bile acids sum, five lipids and two enzymes were observed.

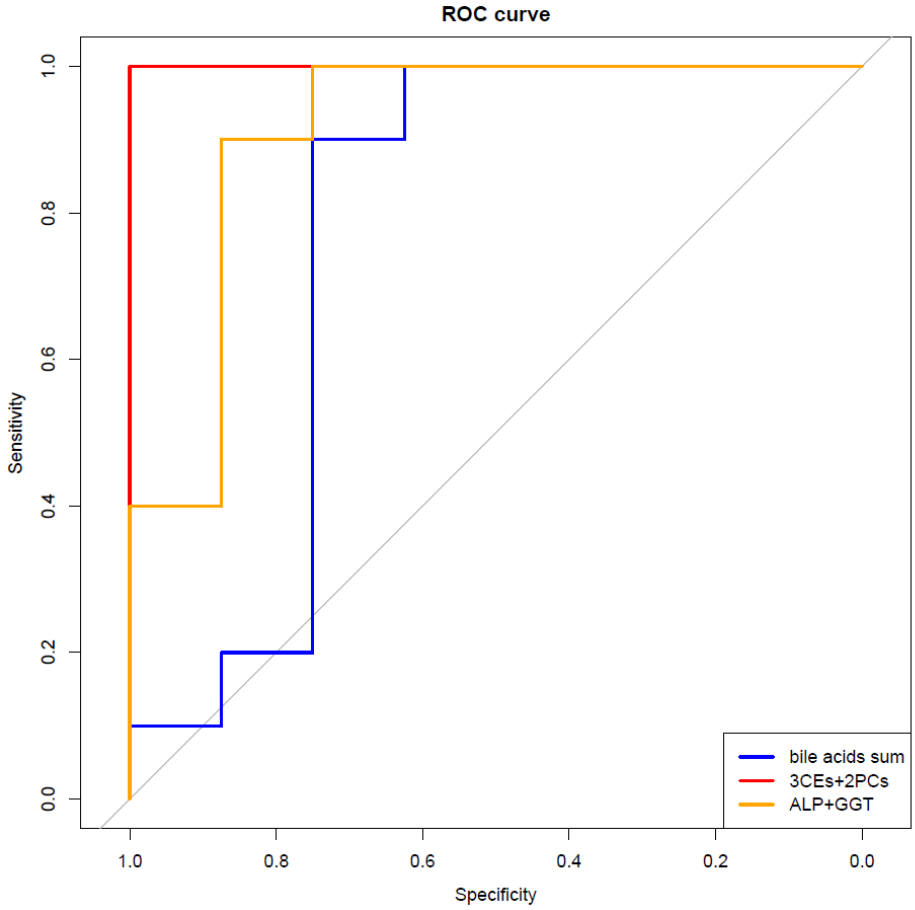


Figure 5.11. ROC curve for comparison of bile acids sum, 5 lipids and plasma enzymes. Bile acids sum, 5 lipids and plasma enzymes resulted in AUC of 77.5%, 100% and 91.2% respectively. CE cholesterol ester; PC, phosphatidylcholine; ALP, alkaline phosphate; GGT, gamma-glutamyl transferase.

5.4 Discussion

Here, a combination of untargeted lipidomics approach and semi- targeted bile acid method using LC-MS was employed to investigate the changes in liver and plasma of CA induced cholestatic mouse.

The outcomes of liver and plasma biochemistry test revealed significant hepatic injury, featuring accelerated serum enzymes levels and enlarged liver weight/BW ratio (**Figure 5.3**), caused by CA supplementation in mice [461, 464].

Although 15 standards were tested in a separate trial, only 8 bile acids were detected. This is possibly related to the fact that the concentrations of some bile acids are below the limit of detection of the applied instrument. By applying semi-targeted analysis for 8 bile acids, CA, GCA, DCA, TDCA and the sum in liver showed higher content, specifically increased levels of plasma CA, TCA, DCA and the sum were observed in CA-fed mice. The impaired bile flows result in accumulation of bile acids in liver [458], and lead to a build-up in plasma and in the circulation. Consistently, we observed elevated levels of bile acids (BAs), including primary and secondary bile acids, in both liver and plasma samples of CA-fed mice. In addition to their detergent effects, accumulation of BAs promotes hepatotoxicity via several mechanisms, including mitochondrial damage, promotion of inflammation, and, ultimately, apoptosis and necrosis [2, 465].

Through conducting multivariate lipidomics analysis, the reproducibility of the experiment was well explained by QCs in the PCA model. As shown in **Figure 5.5a and**

Figure 5.5b, QCs represented in green dots clustered together near the centre of the plot, which indicates the good reproducibility of the sample preparation and analysis.

Correlation analyses were subsequently carried out to find the association between the chosen metabolic features and plasma enzymes. The panel of lipids, mostly PCs and CEs, derived from the multivariate analysis presented to correlate with hepatic injury markers (**Figure 5.8 and Figure 5.9**). Furthermore, the followed univariate analysis of selected common lipids in liver revealed the accelerated contents of three cholesterol esters and two phosphatidylcholines in cholestatic mice (**Figure 5.10**).

Cholesterol esters are derived from cholesterol, which is mainly synthesized in the liver. They then can be transported to the plasma by lipoproteins [466]. A study revealed that CE (18:1), CE (16:0) and CE (16:1) account for about 24%, 12% and 3% of the total fatty acids measured in human plasma cholesterol esters respectively [467]. On top of that, the structure and functionality of all membrane systems are maintained by phospholipid bilayers. Furthermore, phosphatidylcholine is the most abundant phospholipid of eukaryotic membranes [468]. PC (34:2) was found to be up regulated in tissue and serum of various diseases, including lung, colorectal, gastric and thyroid papillary cancer, while increased expression of PC (36:3) was observed to be related with alcohol liver injury [469-471].

The excretion of PC is critical, accumulated PC could facilitate the bile toxicity by attenuating the formation of mixed micelles with BAs and cholesterol [8, 472]. It has also been found in other studies that the cholesterol ester transfer activity was reduced in patients with liver disease and cholestasis [473], which in turn may induce the

gathering of CE [474]. Although the same trend of CEs alteration was observed in plasma, PC levels decreased in the disease group. In healthy controls, most of the circulating CE are formed in plasma through the cholesterol acyltransferase (LCAT) reaction, where LCAT transfers fatty acids from PC to cholesterol through high density lipoprotein (HDL)[474]. With the higher concentration of both HDL and LCAT detected in plasma of human and rodent with hepatic disease [473, 475], plasma PC levels declined in cholestatic mice as seen in this study (**Figure 5.12**).

With the aim of broaden the pool of reliable markers for cholestasis diagnosis, the prediction ability of the selected five lipids, total bile acids examined in this study and the combination of two plasma enzymes ALP, GGT were compared using ROC curve (**Figure 5.11**). The AUC for these three different classes of markers demonstrated that the chosen five lipids could be employed, if reproduced in humans, as a novel tool for cholestatic disease diagnosis.

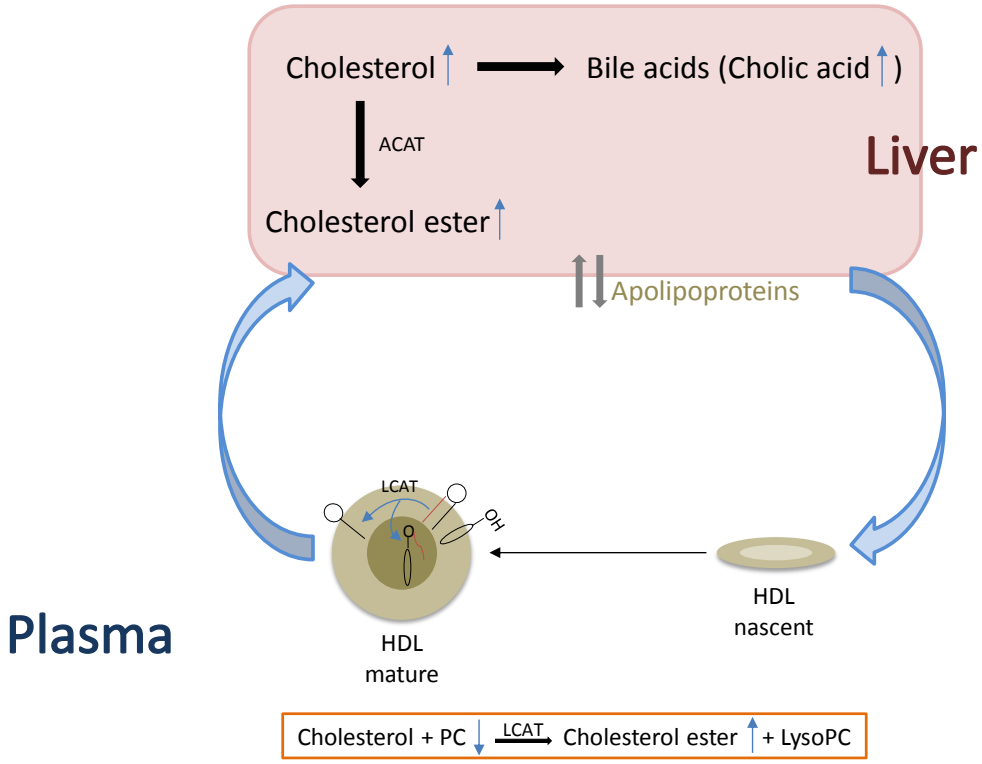


Figure 5.12. Graphic illustration of cholesterol, cholesterol ester and phosphatidylcholine transportation between liver and plasma. ACAT, acyl-CoA cholesterol acyl transferase; HDL, high-density lipoproteins; LCAT, cholesterol acyltransferase. PC, phosphatidylcholine; LysoPC, lysophosphatidylcholine.

5.5 Conclusion

In this preliminary study, the main finding to emerge is that a combination of five lipids including three phosphatidylcholines and two cholesterol esters showed significant differences in terms of concentrations between cholestatic mice and the controls. Also, the panel of lipids correlated well with plasma enzymes. By comparing the plasma lipid metabolites with traditional cholestatic indicators of enzymes and bile acids, better prediction ability was observed. Hence, this panel of lipids have good potential to enhance the selectivity and accuracy of diagnosing cholestasis.

Chapter 6 Metabolomics and Machine learning applied to the septic mouse model

The named researchers below contributed to this chapter by conducting the following experiments.

1. Dr Elizabeth Soares Fernandes (Universidade CEUMA, Brazil): mouse model design, sample/data collection and analysis.
2. Dr Khadija Alawi (King's College London, UK): sample collection and analysis.
3. Dr Aisah Aubdool (King's College London, UK): sample collection and analysis.
4. Mr Pratish Thakore (King's College London, UK): breeding of mice.
5. Mr Saulo José Figueiredo Mendes (Universidade CEUMA, Brazil): sample collection and analysis.

6.1 Introduction

Sepsis, induced by infection, can cause physiologic, pathologic, and biochemical abnormalities [476]. Recently, the new definition of sepsis has been refined as 'life-threatening organ dysfunction caused by a dysregulated host response to infection' [155]. There has been a rapid increase of sepsis cases worldwide, claiming approximately 6 million lives every year [477]. Although enormous improvements in modern medicine have been achieved, only minor progress has been reached in the realm of sepsis as a consequence of insufficient preventive vaccines and therapeutic measures [478].

Despite efforts made for the prompt identification and supportive care of sepsis, including early antibiotics, fluid resuscitation and source control, mortality due to sepsis still ranks second after deaths from heart attack [477, 479]. Surprisingly, recent studies commissioned by the UK Sepsis Trust showed that sepsis affects 260,000 people every year, while long term complications due to delayed diagnosis were found to be costing the UK economy as much as £15.6 billion annually. The fatalities of sepsis in England and Wales increases from 2,136 in 2013 to 2,833 in 2016 [480].

The screening of sepsis can be accomplished by evaluating clinical variables, including Glasgow coma scale score, systolic blood pressure, respiratory rate, serum creatinine and bilirubin, using the criteria of both quick Sequential Organ Failure Assessment (qSOFA) and SOFA which are bedside prompts that may identify patients with suspected infection who are at greater risk for a poor outcome outside the intensive care unit [155]. Furthermore, patients with septic shock can be identified as those with sepsis relying on

vasopressors to maintain mean arterial pressure (MAP) ≥65 mm Hg due to hypotension and with serum lactate levels >2 mmol/L [481].

Since the human body's response to critical blood infection can be identical to its response to other non-infectious triggers [482], it is not easy for clinicians to tell how severe the infection is until organs fail, thus, the discovery of new biomarkers as a tool for aiding early diagnosis and rapid appropriate therapies for patients with sepsis can improve patient outcomes greatly [198]. In 2010, 178 sepsis biomarkers were evaluated in 3370 studies as reported by Pierrakos *et al.* in their review of the literature [199]. From those, biomarkers such as procalcitonin, presepsin, C-creative protein (CPR), CD64, soluble-urokinase-type-plasminogen-activator-receptor (suPAR), soluble triggering receptor expressed on myeloid cells 1 (sTREM-1), IL-6, IL-27, cell-free plasma DNA (cfDNA) and miRNAs have attracted extensive interests [200-202].

Studies applying genomics and proteomics provided a rationale for researchers to consider assessing broader combinations of biomarkers that may improve the real-time diagnostic and prognostic capabilities [198, 483-485]. To learn more about biomarkers in sepsis, a murine model of sepsis has been investigated by utilising metabolomics in this study to obtain a panel of metabolites that would enhance the limitations of individual biomarkers.

6.2 Methods

6.2.1 Reagents

Solvents used for LC-MS analysis including water, methanol and methyl tertiary butyl ether (MTBE) were LC-MS grade purchased from Sigma-Aldrich (Poole, UK). Ammonium formate and internal standards triglyceride (45:0) ($\geqslant$ 98% purity), were purchased from Sigma-Aldrich. All other drugs and reagents were from Sigma-Aldrich (Dorset, U.K.) unless otherwise stated.

6.2.2 Mice

Animals were housed under temperature (22±2˚C)-controlled colony rooms maintained under filtered positive pressure ventilation on a 12-12 h light/dark cycle beginning at 07:00 GMT with free access to water and food. Male, 129S1/SvImJ wildtype (WT) littermates were used at 8-12 weeks of age. All experiments were conducted under the guidelines of the United Kingdom Home Office Animals (Scientific Procedures) Act 1986 and were approved by the King's College London Animal Care and Ethics Committees. All experiments were conducted in a blinded manner. Animals were randomly assigned to control or treatment groups.

6.2.3 Plasma/liver/brain biochemistry

Mice received a single intraperitoneal (i.p.) injection of phosphate buffered saline (PBS) containing lipopolysaccharide (LPS, 11.25 million of EU/kg obtained from *Escherichia*

coli Serotype 111: B4) or vehicle (PBS, 10 ml/kg). For 10 mice in total, 6 received LPS and were classified as septic mice, and 4 were allocated as control mice (vehicle-treated). After 18h, mice were anaesthetised with isoflurane (2-3% carried in 2-3% O2). Blood samples were obtained via a cardiac puncture, using a heparinised syringe and needle (100 U/ml), in the meanwhile, a laparotomy was performed for collection of the peritoneal lavage fluid (PELF). For this, the peritoneal cavity was washed with 3 ml of PBS. Plasma was separated by centrifugation (400×g, 20 min), snap frozen in liquid nitrogen and stored at –80˚C with PELF until processing. Due to the sample volume limitation, plasma was stored for examining biochemistry parameters, and only whole liver and brain were weighed and snap frozen in liquid nitrogen for further metabolomics analysis. Body weight, blood pressure and temperature recordings were taken prior (baseline) and 18h post-LPS. Plasma aspartate aminotransferase (AST), glucose and peritoneal cytokine levels (interleukin (IL) -6, IL-10, tumour necrosis factor-alpha (TNF-α), keratinocyte-derived chemokine (KC) were measured according to manufacturer's instruction Severity scores of sepsis were also evaluated for each mouse [486].

6.2.4 Metabolomics analysis

Sample treatment procedures for mouse liver and brain were conducted as depicted in **Chapter 2.2.5**. For the extracted samples, ether phase was analysed under the same conditions stated in **Chapter 2.2.5**, while the investigation of aqueous phase was conducted following details previously provided (see **Chapter 4.2.3**). All extracted samples were stored at -80 °C before injection. Mass spectrometric conditions for ether

phase analyses were consistent as described in **Chapter 2.2.5**, while the same conditions were applied for aqueous phase analyses as listed in **Chapter 4.2.3**.

6.2.5 Data processing and analysis

Data pre-processing was performed within 'XCMS' package in 'R Studio' (version 1.0.153) and exported into SIMCA version 13 (MKS Umetrics AB, Sweden) for multivariate analysis. Data matrix of RP (+), RP (-), HILIC (+) and HILIC (-) modes from liver and brain samples were processed with normalisation, transformation steps respectively, features with relative standard deviation (RSD) < 30% in QC samples were chosen from each dataset. The combination of selected features from the same phase of the extracted samples (liver RP, liver HILIC, brain RP, brain HILIC) were used for the following model analysis. All model analyses were conducted in R-Studio 1.0.153 (Boston, MA, USA).

Random forest (RF) analysis (500 trees), was conducted to identify metabolites that could distinguish septic and control mice using 'randomForest' package. Contributions of individual predictors were measured by 'variable importance' score. Variable importance scores greater (less) than 0 suggests an increase (decrease) in prediction accuracy. The top 20 variables in variable importance score (> 0) list in descending order were selected in individual models.

Elastic net regression, a type of regularised, or penalized logistic regression (RLR) analysis was applied for binary outcome (sepsis or control) using 'glmnet' package. Significant variables (non-zero coefficients) were shown based on *p*-values in output summary using the minimum lambda.

Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) models were built in SIMCA 14 (Umetrics, Sweden) to compare control and septic mice. Feature selection was done through investigating variable importance projection (VIP) scores and s-plots, the common features from these two platforms (top 50) were chosen.

As mentioned above, three statistical models including random forest, regularised logistic regression and OPLS-DA were built for comparison of sepsis and control groups. Overlapping features from any two models were chosen and combined for four analytical modes. Power analysis, FDR (false discovery rate) control and correlation with biological data were conducted for liver and brain variables separately. Details of the study work flow can be seen in **Figure 6.1**.

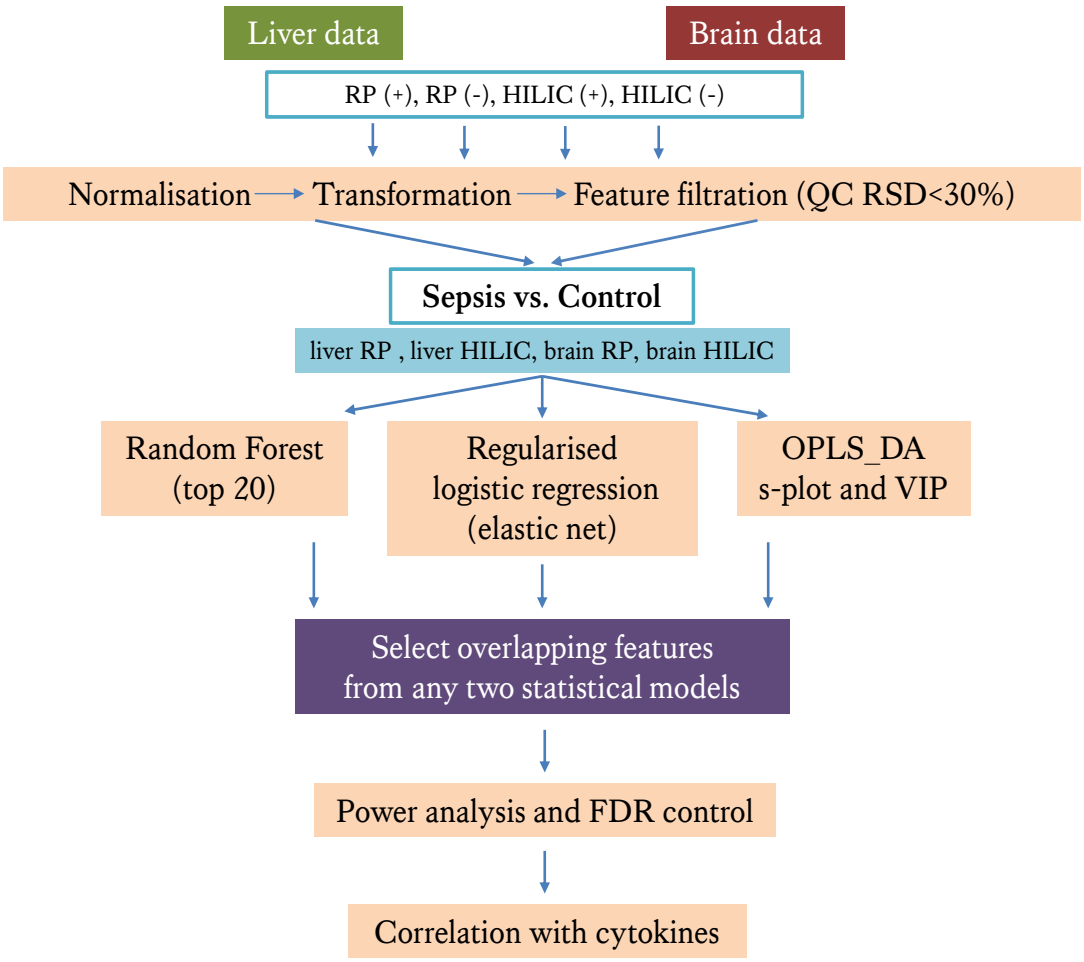


Figure 6.1. Data analysis workflow for mouse liver and brain in a septic model. RP, reverse phase; HILIC, hydrophilic interaction chromatography; OPLS-DA, orthogonal projections to latent structures-discriminant analysis; VIP, variable importance projection; FDR, false discovery rate.

The annotation (level 2 confidence) was performed by comparing fragmentation patterns in the MS^2 data and retention times with in-house and online database (HMDB). All annotated features were measured in the LC-MS data using Waters MassLynx software (Waters Corporation, Milford, MA). Peak areas were normalized to internal standards, and results are expressed as normalised area.

6.3 Results and Discussion

6.3.1 Systemic and biochemistry outcomes

Details of systemic and biochemistry parameters are listed in **Table 6.1**. Fever/low temperature and hypotension are common characteristics of sepsis, and cytokine tests have been widely applied for further diagnosis. Changes in body temperature, blood pressure and body weight after LPS injection can be seen in the septic group. Significant differences were observed between sepsis and control groups for all other outcomes except AST levels. Pro-inflammatory cytokines IL-6, and TNF-α; and anti-inflammatory cytokine IL-10 were employed as biomarkers in the context of sepsis severity for evaluation purpose, and they have been demonstrated with high prediction ability [487, 488]. KC, a chemoattractant for neutrophils, up-regulated in septic mice as it plays a critical role in the course of systemic inflammation [489]. From the comparison above, it shows that mice were septic after LPS induction.

Table 6.1. Systemic and biochemistry outcomes in control and sepsis groups.

Outcomes	Control	Sepsis
Severity score	4.00 (0)	8.50 (0.55) *
Drop in body temperature (%)	-0.28 (1.22)	9.64 (7.32) *
Drop in blood pressure (%)	-16.18 (4.84)	54.94 (11.03) *
Drop in body weight (%)	2.56 (3.98)	12.84 (11.19) *
Glucose levels (mmol/l)	12.42 (2.31)	3.82 (1.73) *
Plasma AST levels (milliunits/ml)	122.10 (32.25)	138.34 (46.12)
PELF IL-10 levels (pg/ml)	254.45 (6.63)	309.66 (29.70) *
PELF TNF-α levels (pg/ml)	46.95 (5.34)	68.58 (14.03) *
PELF IL-6 levels (pg/ml)	1338.70 (137.98)	9822.33 (4822.91) *
PELF KC levels (pg/ml)	253.53 (153.66)	3707.86 (1644.94) *

AST, aspartate aminotransferase; PELF, peritoneal lavage fluid; IL, interleukin; KC, keratinocyte-derived chemokine. Results were expressed as mean (SD), *p*-value was derived from Mann-Whitney test (*p<0.05).

Although these are standard markers of sepsis, they can also be used as markers for other inflammatory disease, such as diabetes, atherosclerosis and Parkinson's disease [490-492].

6.3.2 Data pre-processing

More than 20,000 features were detected using UPLC-Q-ToF under the stated chromatographic and mass spectrometric conditions. Raw data files were processed with 'xcms' package in R, quantile normalisation and log transformation were applied to the four data matrixes.

QC samples were investigated to select features with RSD less than 30%. For liver RP positive data, 2383 out of 4861 features were picked, while 568 out of 664 features met the criteria in negative data. For liver data derived from HILIC positive mode, 1840 out of 2862 features were chosen, while 1161 out of 2944 features from the HILIC negative mode met the standard. Comparatively, for brain RP positive profiles, 2989 out of 3941 features had RSD less than 30%, and 955 out of 1120 fingerprints were selected from brain RP negative data. For brain data obtained in HILIC positive mode, 1886 out of 2615 features were picked, and 1655 out of 2492 features passed the requirement of RSD<30% in negative mode.

With the application of pre-processing steps, the resulting Principal Component Analysis (PCA) score plots showed that QC samples clustered near the origin suggesting features were robustly detected and reproduced during the analysis. Plots for each dataset mentioned above can be seen in **Figure 6.2.**

Table 6.2. Feature selection with RSD value in QC samples.

Dataset	Total	RSD < 30% in QC samples
Liver RP (+)	4861	2383
Liver RP (-)	664	568
Liver HILIC (+)	2862	1840
Liver HILIC (-)	2944	1161
Brain RP (+)	3941	2989
Brain RP (-)	1120	955
Brain HILIC (+)	2615	1886
Brain HILIC (-)	2492	1655

RSD, relative standard deviation; QC, quality control; RP, reverse phase; HILIC, hydrophilic interaction chromatography.

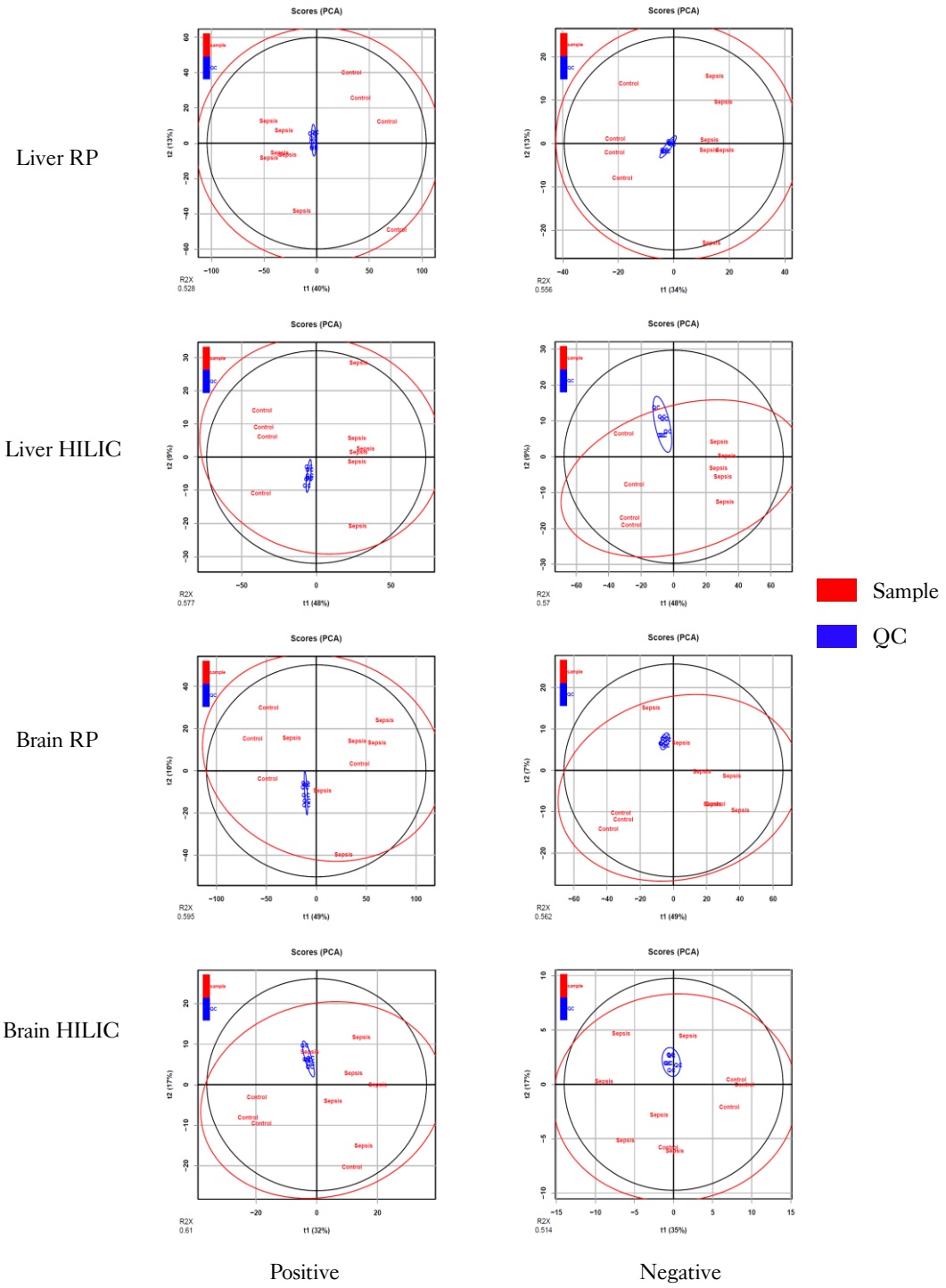


Figure 6.2. PCA plots of liver and brain data with different chromatographic and mass spectrometric conditions. Colour red represents samples, and colour blue represents QCs.

6.3.3 Model analysis

The Random forest (RF) approach was used to identify the top 20 lipids, in terms of their importance score in each dataset after pre-processing. RF is a versatile classification algorithm which can capture complex and non-linear relationship between predictors and outcomes [493]. It differs from PCA (a dimensionality reduction algorithm), which identifies the hyper-plane closest to the data and projects the data onto it, instead the RF method relies on the assembling of decision trees built with random selection of features. This machine learning method provides two aspects that are very informative for data mining: high prediction accuracy and information on variable importance for classification [494].

By applying the elastic net regression (regularised logistic regression), variables with non-zero coefficients were extracted from the output. Elastic net regression is a hybrid approach that blends both penalization of the lasso and ridge regression while separating the two groups with minimizing mean-squared error (MSE). As an approach for prediction modelling, it is suitable for both large data sets (bioinformatics, neuroimaging) and data sets with high dimensionality and small sample size like the dataset in this project [495, 496]. From the statistical perspective, elastic net regression controls for the effects of the overfitting and multicollinearity [497].

OPLS-DA models were built for all four combined datasets. For liver RP data (R^2X=0.624, Q^2=0.96), CV-ANOVA p value (1.08×10^{-2}) suggested the model to be reliable, and the R^2Y= 0.995 in score plot indicated a good discrimination between septic and control mice. Similarly, CV-ANOVA p value of 2.43×10^{-4} and the R^2Y value of 0.994 showed a clear separation of two groups in liver HILIC data (R^2X=0.791,

Q^2=0.978). For brain RP data (R^2X=0.742, Q^2=0.577), the CV-ANOVA *p* value of 2.84×10^{-2} and the R^2Y value of 0.955 demonstrated segregation between groups. CV-ANOVA *p* value (2.23×10^{-2}) in brain HILIC data (R^2X=0.458, Q^2=0.623) explained the model to be valid, and the R^2Y= 0.969 in score plot revealed a good discrimination.

Overlapping features from any two of the above-mentioned data treatment models were selected. As shown in **Figure 6.3**, there were 31 features chosen in liver data, including 13 from RP, and 18 from HILIC mode. Similarly, a total of 23 features were selected in brain data, with 12 from RP, and 11 from HILIC condition.

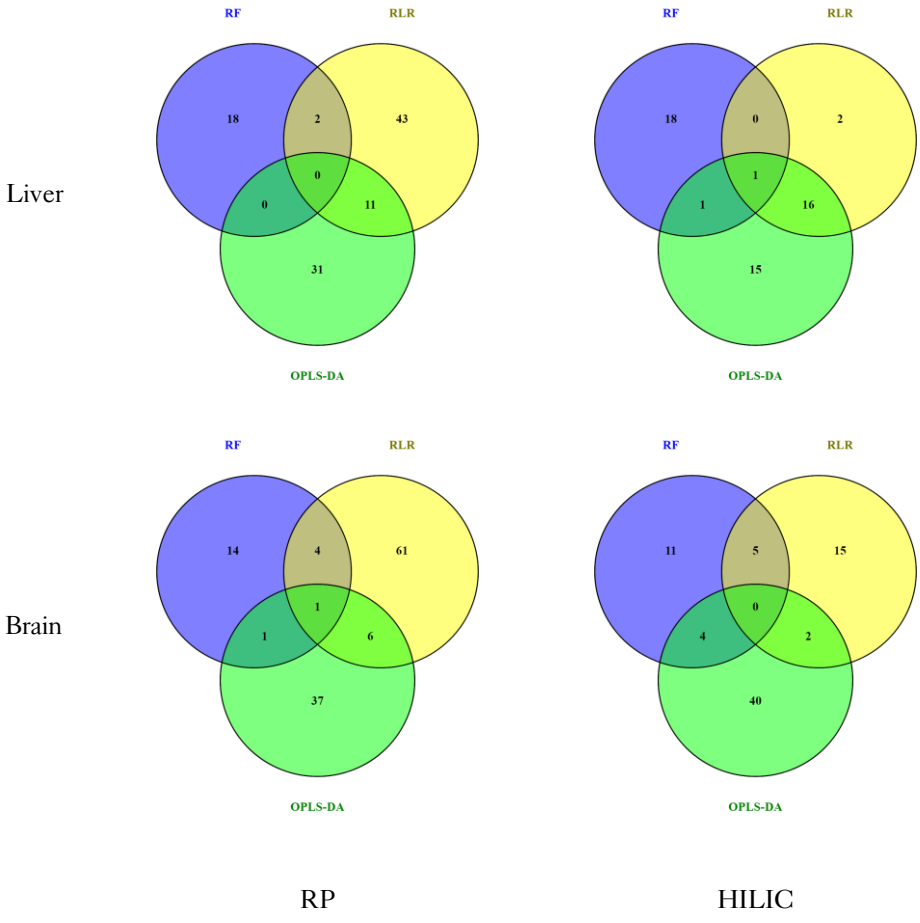


Figure 6.3. Venn Diagrams show overlapping features from three statistical models in liver and brain data.

6.3.4 Univariate analysis

From all 54 selected features shown in the Venn diagram, 20 metabolites were annotated. The annotations of the metabolites can be found in **Table 6.3**. Due to the limitation of the small sample size in this study, post hoc power analysis (alpha=0.05, power=0.8) was applied to examine the sample size needed for each annotated metabolite. From 20 annotated metabolites, 9 passed the power analysis threshold, where the differences between septic and control groups can be detected with the quantity of samples employed in this study. Additionally, Benjamini- Hochberg correction was conducted for FDR control following the Mann- Whitney test between control and sepsis groups on 9 metabolites. Plots of these metabolites including 3 cholesterol esters (CE), 2 phosphatidylethanolamines (PE), lysophosphocholines (LysoPC) (16:0), pyruvate, fumarate and glycerylphosphorylethanolamine (GPE) can be seen in **Figure 6.4**. Results were presented as mean ± SD, QC samples were also included in the bar plots to easily compare the analytical variation of chosen metabolites.

Significant differences were observed in the levels of CE (18:1), CE (16:0), CE (18:2), LysoPC (16:0), pyruvate, fumarate and GPE in liver between control and sepsis groups. Comparatively, the content of CE (18:2), PE (p38:0) and GPE showed differences in the brain profile. Among these 9 metabolites, pyruvate and fumarate showed opposite trends in liver and brain regarding their concentrations when comparing sepsis and control samples.

Both CE (18:1), CE (16:0) exhibited increased levels in the liver and plasma of cholestatic mice as studied in **Chapter 5**, whereas studies have shown that sepsis syndrome can lead to a disbalance of lipid homeostasis [498, 499]. Cholesterol

accumulation induced by hypercholesterolaemia promotes inflammatory responses in conditions like sepsis [500]. However, the stimulated inflammatory response could also lead to decreased cholesterol efflux [501], which results in reduction of cholesterol esters, a downstream product of cholesterol, as shown in **Figure 6.4**.

Table 6.3. Annotated metabolites from the common features in three statistical models.

	Metabolites	Retention time (min)	mass to charge ratio (m/z)	Detection mode
Liver	CE (18:1)	29.57	668.64	RP
	SM (40:2)	23.97	786.66	RP
	CE (16:0)	28.84	642.62	RP
	CE (18:2)	27.96	666.62	RP
	LysoPC (16:0)	3.34	496.34	RP
	AMP	16.83	346.05	HN
Brain	PE (40:5)	16.96	792.57	RN
	PE (p38:0)	19.76	818.64	RN
	PE (33:1)	16.35	762.53	RN
	PC (35:4)	18.13	766.55	RN
	PE (38:4)	16.45	766.55	RN
	PE (p36:2)	17.71	772.54	RN
	PE (42:4)	17.41	804.59	RN
	PI (38:4)	16.02	885.57	RN
	PE (46:4)	19.55	860.65	RN
	PE (p40:0)	20.02	846.67	RN
	Pyruvate	11.45	87.01	HN
	Fumarate	11.46	115.00	HN
	GPE	16.76	214.05	HN
	Phosphoric acid	13.36	96.97	HN

CE, cholesterol ester; SM, sphingomyelin; LysoPC, lysophosphocholines; AMP, adenosine monophosphate; PE, phosphatidylethanolamine; PC, phosphocholine; PI, phosphatidylinositol; RP, reverse phase positive; HN, HILIC negative; RN, reverse phase negative.

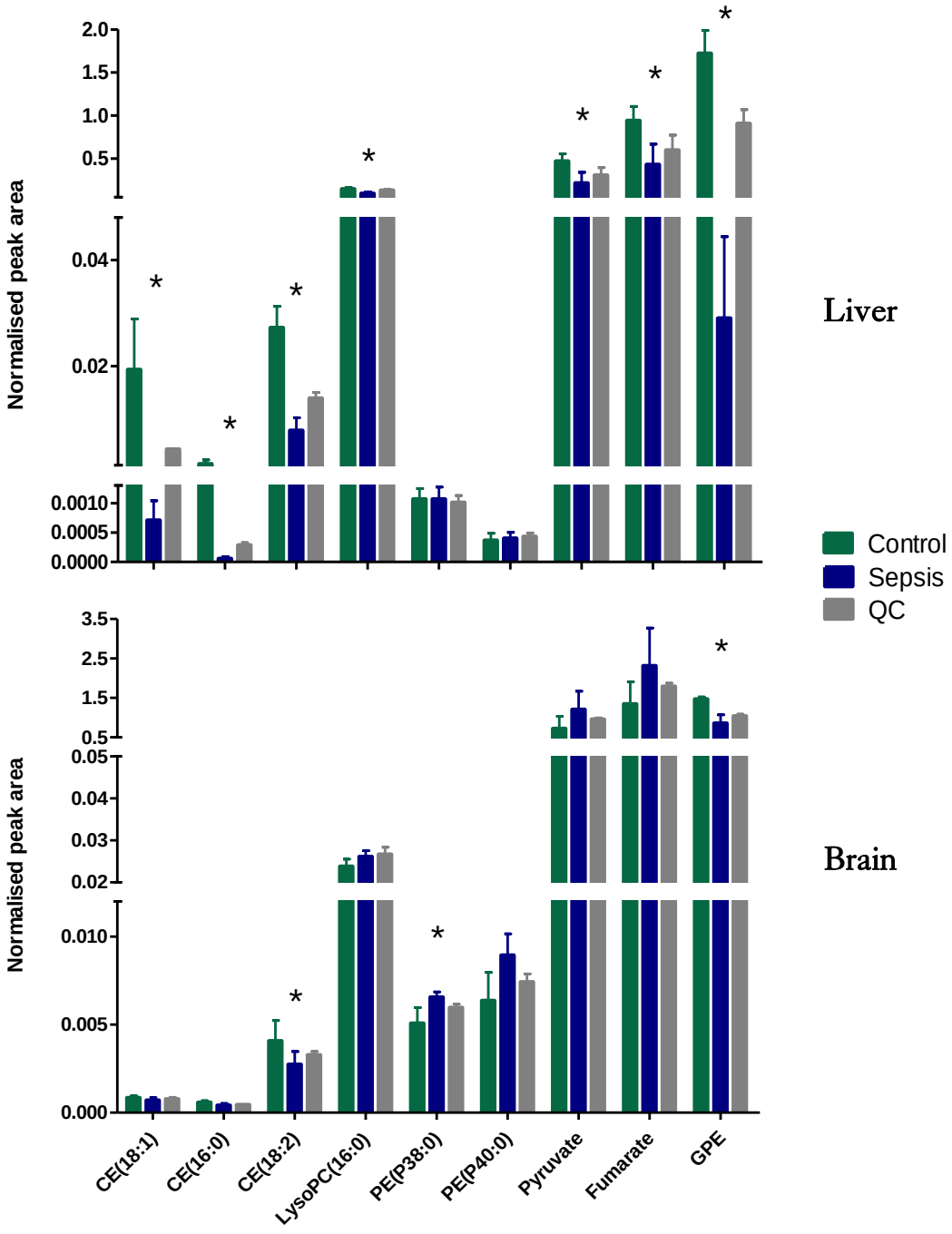


Figure 6.4. Bar plots of 9 metabolite profiles in liver and brain samples. Normalised peak area was illustrated as mean ± SD, *p*-values were derived from Mann-Whitney test followed by Benjamini-Hochberg correction (**p*<0.05, ***p*<0.01, ****p*<0.001).

Interestingly, LysoPC (16:0) has been investigated in **Chapter 2**, and higher amount in the liver tissue of donation after circulatory death (DCD) patients was observed when compared with donation after brain death (DBD) patients where raised levels of pro-

inflammatory changes were also discovered [54]. Additionally, it presented elevated level in the group of patients who showed early allograft disfunction (EAD) after liver transplantation. Furthermore, decreased plasma concentration of various LysoPC species including LysoPC (16:0) was observed in a study involving 102 sepsis patient and 56 healthy controls [502], which is consistent with the findings in this chapter.

Fumarate concentration significantly diminished in the liver of sepsis mice (**Figure 6.4**). This could be associated with a biochemical mechanism within the mitochondria where the oxidation of succinate to fumarate is inverted in sepsis, leading to fumarate reduction [503]. In addition, the TCA cycle where fumarate participates might have been hampered by LPS injection in our sepsis model, which can trigger the blockade of the TCA cycle [504]. Similarly, the apparent decline of pyruvate suggests an inhibition of glycolysis where pyruvate is the end product [504]. Although their levels elevated in brain profiles, it could be linked with an enhanced energy metabolism in brain with septic conditions [505, 506]. Heath *et al.* found that hyperdynamic state was observed in sepsis patients for weeks or even months, where the energy demand was increased [507]. In addition, it was observed that sepsis did not hinder the ability of brain to maintain high energy metabolism during hypoxia in rats [508].

Phosphatidylethanolamine, one of the most studied phospholipids in the context of cell death, can flip from the inner plasma membrane to the outer membrane during inflammation inducing cell death dynamics [509, 510]. With the accumulation of PE after cell death, significantly elevated amounts of PE were observed in septic mice brain tissue. As the intermediate in the catabolism of PE [511], GPE (**Figure 6.5**) concentration was lower in both septic liver and brain samples. This lipid was

demonstrated to be essential for anticoagulation activity, and its altered levels could be associated with coagulation and vascular endothelial dysfunction during severe sepsis [512, 513].

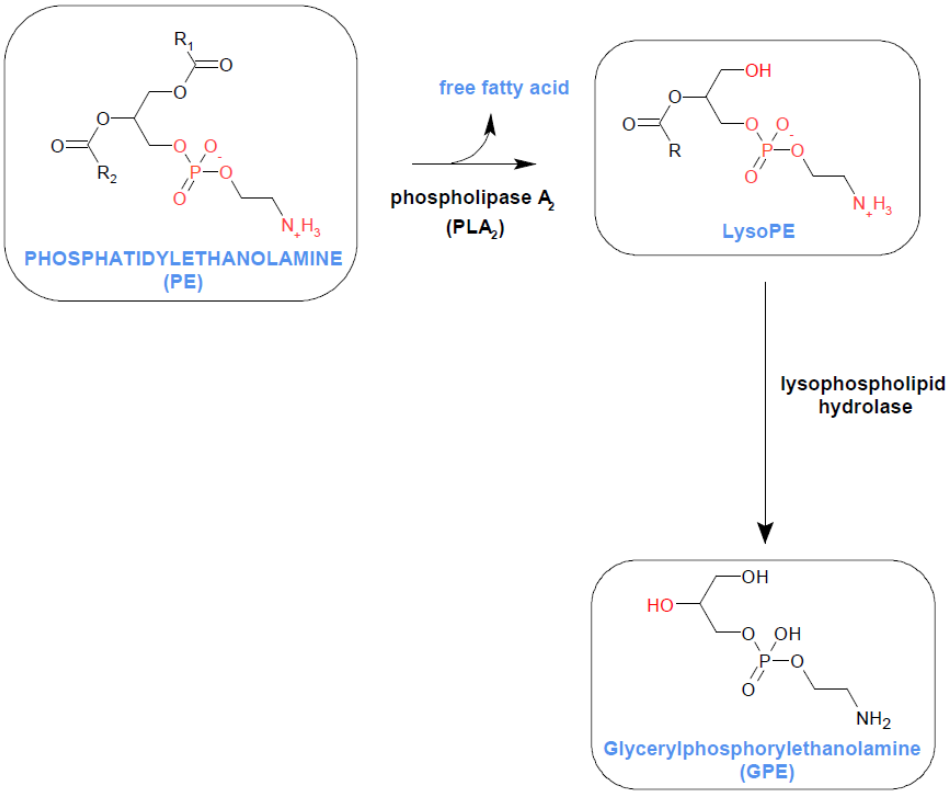


Figure 6.5. Pathway of glycerylphosphorylethanolamine (GPE) from PE.

6.3.5 Correlation analysis

Spearman correlation analyses between the 9 annotated metabolites, 4 PELF cytokines and systemic response factors (glucose level, drop in body temperature, blood pressure and body weight) were carried out in this study. As shown in **Figure 6.6**, cholesterol

esters were highly positively correlated with each other (all r > 0.9 and p < 0.001) in the liver profile. Strong correlation was also observed between Pyruvate and fumarate, as well as between IL-6 and TNF-α, KC (all r > 0.9 and p < 0.001). Moderate correlations were observed between LysoPC (16:0), pyruvate, fumarate and three CE molecules (all r > 0.7 and p < 0.01). Additionally, negative correlations were discovered between LysoPC (16:0), pyruvate, fumarate, GPE and the four investigated cytokines (all r < -0.65 and p < 0.05) while pyruvate, fumarate and GPE correlated positively with glucose level (all r > 0.75 and p < 0.01).

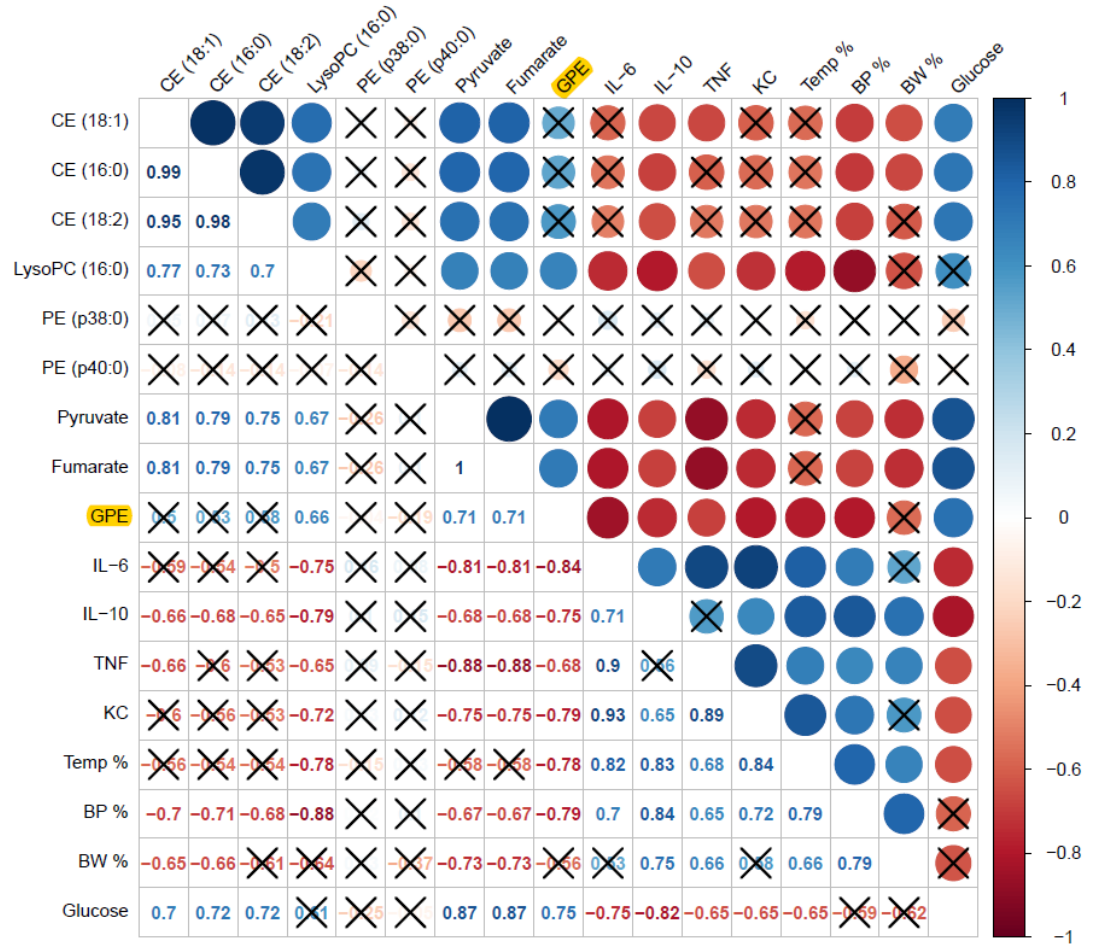


Figure 6.6. Correlation plot of 9 metabolites in liver, 4 PELF cytokines and systemic response factors. Correlation coefficients were listed at the lower part of the plot, blue represents positive correlation while red represents negative correlation. Intensity of 'redness' and 'blueness' represents correlation coefficient value. Cell with X shows correlation below the significant level (0.05).

Correlation analyses for 9 metabolites in the brain profile, 4 PELF cytokines and systemic response factors (glucose level, drop in body temperature, blood pressure and body weight) were illustrated in **Figure 6.7**. Similar correlation matrix can be seen for brain data when compared with liver data. Very strong positive correlation can be seen between Pyruvate and fumarate, GPE and glucose, as well as between IL-6 and TNF-α, KC (all r > 0.9 and p < 0.001). Contrastingly, PE (p40:0) correlated negatively and significantly with CE (16:0) and CE (18:2) (all r > 0.9 and p < 0.001). Moderate correlation between GPE and all four cytokines was observed (all r < -0.7 and p < 0.05).

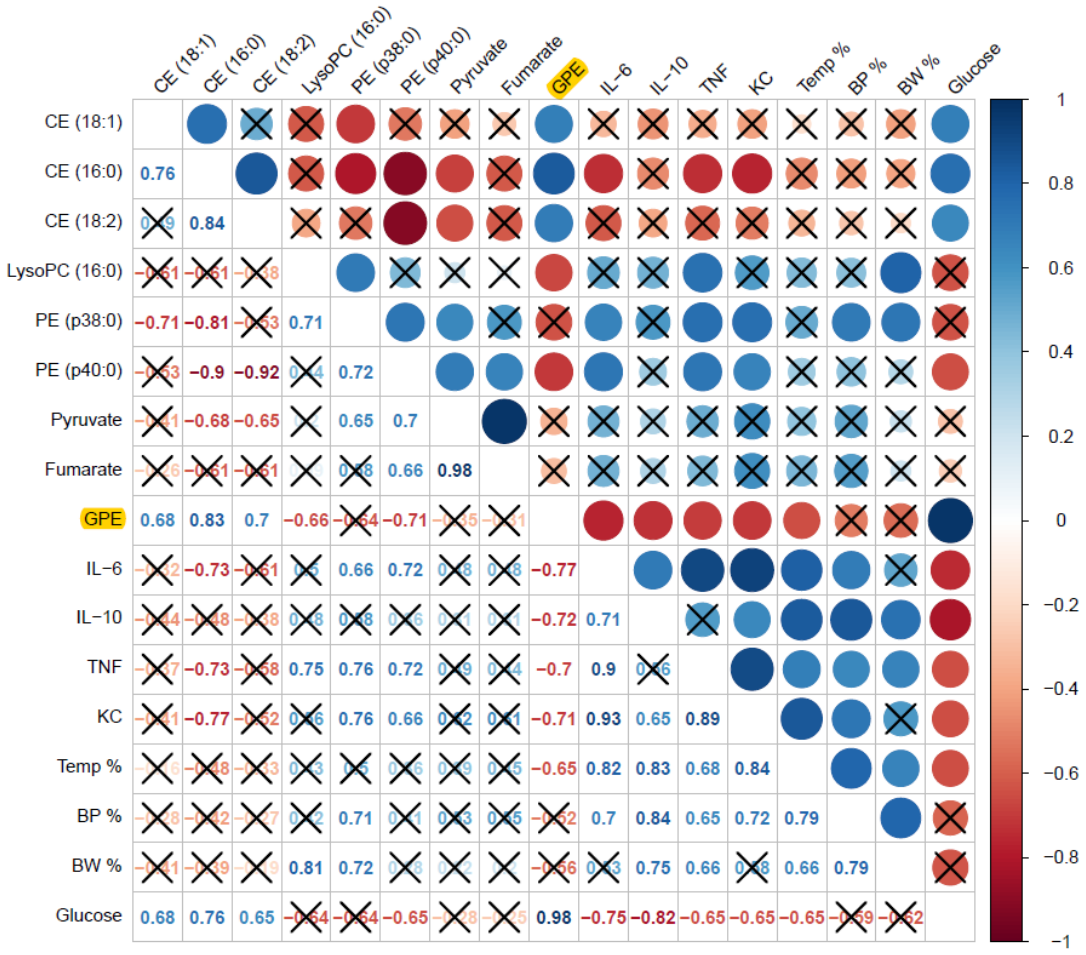


Figure 6.7. Correlation plot of 9 metabolites in brain, 4 PELF cytokines and systemic response factors. Correlation coefficients were listed at the lower part of the plot, blue represents positive correlation while red represents negative correlation. Intensity of 'redness' and 'blueness' represents correlation coefficient value. Cell with X shows correlation below the significant level (0.05).

Among the panel of 9 metabolites, GPE in liver and brain both showed correlation with inflammatory cytokines (-), glucose level (+) and drop in body temperature (-). GPE is involved in maintaining membrane fluidity, and being the essential source of the key components of the phospholipid bilayer, PE and phosphatidylcholine (PC) [514]. It is known to improve the cognitive impairment that occurs in many neurodegenerative diseases, as well as hinder the progress of neural stem cell aging [515, 516].

6.4 Conclusion

In summary, metabolomics approach was applied here to explore potential metabolite indicators for sepsis utilising both mouse liver and brain tissue. With the combination of three multivariate analytical tools including random forest, regularised logistic regression and OPLS-DA, a panel of 9 annotated metabolites was established. Through the correlation analyses, glycerylphosphorylethanolamine was selected as a putative biomarker which was depleted in septic mice and very highly correlated with inflammatory cytokines and systemic response factors of sepsis.

Chapter 7 Final conclusions and future directions

7.1 Final conclusions

Liver related diseases are rapidly becoming a major global burden. Metabolomics can provide a window to view the biochemistry occurring in the transitional phases between a healthy liver and a diseased liver. Whether provoked by diabetes, cholestasis, alcohol use, sepsis or oncogenic viruses, the liver develops a core metabolomic phenotype that involves dysregulation of bile acid and lipid homeostasis. Considering these key changes in metabolism, a metabolomic approach can shed light on the overall biochemistry in the liver and help designing diagnostic and/or progression tests associated with hepato-related diseases. To this end several studies were designed and undertaken in this program of research, and the results were presented and discussed in this thesis. Putative markers identified in each chapter were listed in **Error! Reference source not found.**.

Table 7.1. List of putative markers identified in each chapter.

Liver Diseases	IRI	Allograft Function	Cholate Injury	Inflammatory Response
Chapter 2 Liver Transplantation	LysoPC (16:0) LysoPC (18:0)			
Chapter 3 Liver Transplantation	Cer (36:1)			Cer (36:1)
Chapter 4 Liver Transplantation		AMP Adenine		
Chapter 5 Cholestasis			CE (16:0) CE (16:1) CE (18:1) PC (36:3) PC (34:2)	
Chapter 6 Sepsis				CE (18:1) CE (16:0) CE (18:2) LysoPC (16:0)

		PE (p38:0)
		PE (p40:0)
		Pyruvate
		Fumarate
		GPE

IRI, Ischaemia reperfusion injury; LysoPC, Lysophosphatidylcholine; Cer, Ceramide; AMP, adenine monophosphate; CE, cholesterol ester; PC, phosphatidylcholine; PE, phosphatidylethanolamine; GPE, glycerylphosphorylethanolamine.

In chapter 2, reversed phase-mass spectrometry (RP-MS) was employed to compare lipid fingerprints between 76 DBD (donation after brain death) and 36 DCD (donation after circulatory death) matched pre- and post-transplant liver biopsies. The results suggested that two LysoPCs could play a role in perpetrating tissue damage induced by warm ischaemia. This can be further supported by the fact that higher levels of these two LysoPCs were observed in recipients undergoing early allograft dysfunction (EAD) ($p < 0.05$) when compared with immediate graft function (IGF) group. Additionally, they were also found to correlate with AST concentration at post-transplant. Studies suggest that free fatty acids mediated cytotoxicity in liver diseases, such as NAFLD, is indirect via the generation of the toxic metabolite, LysoPC. Rationally designed therapies can hopefully be developed if LPC-mediated pathways for lipotoxicity can been elucidated [517], Lehmann *et al.* also found that LysoPCs are able to separate the insulin-sensitive from the insulin-resistant NAFLD group in humans and may highlight important pathways in the pathogenesis of fatty liver induced insulin resistance [518].

In chapter 3, we were interested in measuring lipids that are known to induce inflammation since ischaemia reperfusion injury (IRI) is associated with the release of pro-inflammatory mediators. Semi-targeted analysis of 5 ceramide molecules was

carried out in 20 DBD and 26 DCD paired liver samples. Increased levels of C18 and C20 ceramides and diminished levels of C24 ceramide were observed in DBD post-transplant phase compared with pre-transplant phase ($p<0.05$), C22 ceramide showed more pronounced changes in the DCD group. C18 ceramide was found to be associated with post-transplant international ratio (INR) creatinine and bilirubin, three common markers of liver dysfunction. The work in this chapter therefore contributed to the growing body of evidence highlighting the involvement of C18 ceramide in IRI in DCD. Other researchers have revealed that a decrease in C24 ceramide serum levels was associated with severity of liver fibrosis in HCV patients, and was proposed as a promising novel biomarker in chronic HCV infection [519]. It was also reported that increased hepatic long-chain ceramides were associated with apoptosis (C16 and C18) and that decreased very-long-chain ceramide C24 are involved in insulin signalling [520].

In chapter 4, hydrophilic interaction chromatography-MS (HILIC-MS) analysis was performed on 35 DBD and 12 DCD paired liver samples (n=94). Sample subset was divided into training set (30 DBD, 5 DCD) and test set (5 DBD, 7 DCD). Subsequent orthogonal projections to latent structures-discriminant analysis (OPLS-DA) modelling highlighted five purine metabolites, including adenine, adenosine, adenosine monophosphate (AMP), hypoxanthine and uric acid. Elevated levels of AMP and adenine were found in the DBD *vs.* DCD models at both transplant stages ($p<0.01$), after conducting univariate analysis. These changes were also seen in pre-transplant biopsies from recipients experiencing immediate graft function (IGF) ($p<0.05$). In the meanwhile, the prognostic value in terms of receiver operating characteristic (ROC) area under the curve (AUC) for these two metabolites was above 0.70, surpassing the

values of traditional enzyme markers. The findings suggested that they may associate to healthy allograft function and a test could improve graft survival rate. Interestingly, it was reported that the activation of AMP-activated protein kinase in liver decreased lipogenesis and protected against hepatic steatosis when mice were fed a high-fructose diet. It is also protective against hepatic triglyceride accumulation, which leads to NAFLD [521].

In chapter 5, now in animal models, untargeted lipidomic analysis and semi-targeted analysis of 8 bile acids were performed on 6 cholestatic and 7 control mouse liver samples, as well as on 10 cholestatic and 8 control mouse plasma samples in search of cholestasis biomarkers. A combination of 15 liver lipid features and 13 plasma lipid features consisting of cholesteryl ester (CE), triglycerides (TG) and phosphatidylcholines (PC) was identified and the correlation analyses among this panel and 5 plasma enzymes were conducted. In addition, common features from liver and plasma matrixes demonstrated close correlations with enzymes were selected for further univariate analysis. Two PCs and three CEs showed significant differences between cholestatic and control mice (p<0.05). Further, ROC curve revealed a high potential of these five lipids in discriminating cholestatic mice from controls, with AUC of 100%, when compared to plasma enzymes (91.2%) and bile acids (77.5%). Cholesterol and PC can form CE and LysoPC with the assistance of lipoproteins, the changes of these lipids can be used as biomarkers of the degree of cholestasis, with serum cholesterol being an even more sensitive marker of cholestasis among early-stage PBC patients than serum bilirubin [522].

In the last chapter, the interest shifted toward sepsis in order to understand inflammation in the liver and the connection between liver-brain axis. Liquid Chromatography-Mass Spectrometry (LC-MS) was applied on liver and brain biopsies from 6 septic and 4 control mice. To maximize detection of metabolite numbers, two orthogonal LC methods, PR and HILIC, were employed. This experimental design allowed detection of more than 20,000 metabolic features. A panel of 9 annotated metabolites, including previously discussed LysoPC (16:0), CE (16:0) and CE (18:1), were uncovered through a meta-analysis which employed three machine learning tools. Mann -Whitney tests and spearman correlation analyses revealed glycerylphosphorylethanolamine (GPE) depleting in both liver and brain from septic mice (p<0.05) and correlating strongly with conventional sepsis markers. It implicated that GPE can act as a candidate biomarker for endothelial dysfunction during sepsis.

In summary, chapter 2, 3 and 4 supported the general hypothesis in liver transplantation, addressing the practical possibilities of novel biomarkers being tested in the clinic. In chapter 5 and 6, the metabolomics strategy was applied to two mouse models of liver related diseases. Overall, this thesis has shown that, metabolic profiling techniques of various biological samples have a practical application in the discovery of metabolic signatures associated with a number of liver related diseases. Nowadays, personal medicine has been a new dimension in patient care with the power of disease diagnosis, prognosis and therapeutic response monitoring. Metabolomics which provides a global view of health with integrated output of multiple systems and organs can be a valuable approach for the development of stratified medicine [4].

7.2 Future directions

7.2.1 Further validation of markers

In Chapter 2, 3 and 4, we were able to identify a few liver tissue metabolic features associated with organ damage, inflammation or function. However, liver biopsies are not easily accessible in clinic. Additional validations of these markers with donor blood samples would facilitate the evaluation process. Furthermore, since both normothermic machine perfusion and living donation (LD) have been demonstrated to show reduced level of graft injury, measurements of these markers from normothermic perfused and LD biopsies are of great interest.

In chapter 5 and 6, animal models of cholestasis and sepsis, respectively, consisted of small sized sample cohorts. Therefore, another interest for the future work involving animal samples would be to validate metabolites in a separate larger-scale sample set.

It is plausible to work towards conducting a clinical trial of two LysoPC biomarkers which were found to be associated with liver damage, and to ascertain how to supplement the transplanted livers with AMP and adenine which were are depleted, in order to improve transplant outcomes.

7.2.2 Metabolite identification

One of the major challenges in metabolomics studies is the identification of metabolites. Compared to peptides consisting of amino acids repeatedly arranged in linear orders, metabolites are random combinations of some basic elements (e.g. C, H, O, S, N, and P). Moreover, the chemical and physical diversities of metabolites make them difficult to be identifed based on MS non-targeted approach. In Chapter 2, 4, 5 and 6, we examined metabolic fingerprints obtained by LC-MS untargeted methods and found numerous metabolic features which have shown to be altered significantly between sample cohorts. While we were able to annotate some of them, they need to undergo strict experimental verification procedures. Authentic standards need to be obtained and analysed under the identical experimental conditions with the biological samples. For identification with high confidence level, mass to charge ratio, retention time and fragmentation spectra between the samples and the authentic standards need to be compared.

Identification of metabolites associated with liver related diseases would also allow us to carry out a secondary analysis, such as enrichment analysis and metabolite mapping, enabling further analysis of metabolomics data. Enrichment analysis computes metabolic pathways that have been significantly altered by using a collection of pre-defined metabolite pathways and disease states obtained from the literature. This analysis can aid identification and interpretation of patterns on metabolite concentration/level changes in a biologically meaningful context. Meanwhile, Metabolite mapping provides a visual representation of metabolomics data by highlighting the identified metabolites (and their abundances) on a network graph, often

obtained from a biochemical database. Therefore, achieving identification of interest metabolites would allow these secondary analyses to be carried out, in turn providing good biological interpretations of the data.

7.2.3 Metabolomics analysis standardisation

Metabolomics-based strategies have become a major part of modern clinical research, allowing for a better understanding of pathophysiological conditions and disease mechanisms, as well as providing innovative tools for more adequate diagnostic and prognosis approaches. Nevertheless, compared to other '-omics' studies, research conducted with metabolomics approach lack the standardisation for sampling protocols, data exchange and data treatment workflow. Examples of such specifications have been proposed by Coordination of Standards in Metabolomics (COSMOS) and The Chemical Analysis Working Group of the Metabolomics Standards Initiative (MSI).

COSMOS develops a robust data frame and exchange standards for metabolomics data expediates the transfer of experimental information between research groups, as well as potentially to publishers and funders. Also, MSI provides a biological and experimental context for the data, enables experimental replication, and facilitates the comparison of data by other researchers. Therefore, there is a great need to apply such specifications in metabolomics field and it is something that we would like to work in the future.

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